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<b>(54) Title:</b> EPITHELINS: NOVEL CYSTEINE - RICH GROWTH MODULATING PROTEINS  <b>(57) Abstract</b> <p>A novel family of growth regulatory proteins termed "epithelins" are described. The epithelins comprise several distinct members sharing significant structural homology. Two members of the epithelin family, epithelin 1 and epithelin 2, have been purified from natural sources. In addition cDNA and PCR clones encoding mature and precursor epithelins from various chordate sources have been obtained and sequenced, including the complete human, mouse and rat epithelin precursors. The recombinant expression of rat epithelin precursor and mature forms is described. <u>Purified epithelin 1 is a bifunctional growth regulator, capable of stimulating the growth of some cell types while inhibiting the growth of others.</u> Purified epithelin 2 is functionally similar to epithelin 1 with respect to growth inhibitory bioactivity. In contrast, however, epithelin 2 is apparently not capable of eliciting the growth stimulatory activity characteristic of epithelin 1 and, in fact, antagonizes this epithelin 1 activity.</p>		

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EPITHELINS: NOVEL CYSTEINE-RICH GROWTH MODULATING PROTEINS1. INTRODUCTION

The present invention relates to a novel family of growth regulatory proteins which applicants have termed "epithelins", to methods for the production of epithelins, and to their diagnostic and therapeutic uses. Applicants have purified two members of the epithelin family, epithelin 1 and epithelin 2, from natural cell sources and have isolated cDNAs encoding several different epithelins. Epithelin 1 and epithelin 2 share substantial structural similarity yet are functionally distinct proteins. Epithelin 1 is a bifunctional growth regulator, capable of stimulating the growth of some cell types while inhibiting the growth of others. Epithelin 2 is functionally similar to epithelin 1 with respect to growth inhibitory bioactivity. In contrast, however, epithelin 2 is apparently not capable of eliciting the growth stimulatory activity characteristic of epithelin 1 and, in fact, antagonizes this epithelin 1 activity.

2. BACKGROUND OF THE INVENTION

Cellular growth and differentiation appear to be initiated, promoted, maintained, and regulated by a multiplicity of stimulatory, inhibitory, and synergistic factors and hormones. The alteration and/or breakdown of the cellular homeostasis mechanism seems to be a fundamental cause of growth-related diseases, including neoplasia. Growth modulatory factors are implicated in a wide variety of pathological and physiological processes including signal transduction, cell communication, growth and development, embryogenesis, immune response, hematopoiesis, cell survival and differentiation, inflammation, tissue repair and remodeling, atherosclerosis and cancer.

Epidermal growth factor (EGF), transforming growth factor- $\alpha$  (TGF $\alpha$ ), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), nerve growth factor (NGF), transforming growth factor- $\beta$  (TGF $\beta$ ), insulin growth factor I and II (IGF I, IGF II), hematopoietic growth factors such as erythropoietin, colony-stimulating factors (CSF 1 and 2), interleukins (IL-1 to 8), interferons (IFN  $\alpha$ ,  $\beta$ ,  $\gamma$ ), tumor necrosis factor  $\alpha$  and  $\beta$  (TNF  $\alpha$  and  $\beta$ ), leukoregulin, oncostatin M, amphiregulin (AR) and other less defined factors are growth and differentiation modulatory proteins produced by a variety of cell types either under normal physiological conditions or in response to exogenous stimuli. Most of these factors appear to act in autocrine and paracrine fashions. (For reviews see: Goustin et al., 1986, Cancer Res. 46:1015-1029; Rozengurt, 1986, Science 234:161-166; Pardee, 1987, Cancer Res. 47:1488-1491; Sachs, 1986, Sci. Amer. 254:40-47; Marshall, 1987, Cell 50:5-6; Melcher and Anderson, 1987, Cell 30:715-720; Namen et al., 1988, J. Exp. Med. 167:988-1002; Baggiolini et al., 1989, J. Clin. Invest. 84:1045-1049; Clemens and McNurlan, 1985, Biochem. J. 226:345-360; Nathan, 1987, J. Clin. Invest. 79:319-326; Sporn and Roberts, 1986, J. Clin. Invest. 78:329-332; Old, 1987, Nature 326:330-331; Beutler and Cerami, 1987, New Engl. J. Med. 316:379-385; Weinstein, 1987, J. Cell. Biochem. 33:213-224; Zarling et al., 1987, Proc. Natl. Acad. Sci. USA 83:9739-9744; Shoyab et al., 1988, Proc. Natl. Acad. Sci. USA, 85:6528-6532; Shoyab et al., 1989, Science 243:1074-1076; Sporn and Todaro, 1985, N. Engl. J. Med. 303:878-880; Sporn and Roberts, 1985, Nature 313:745-747).

There is a great deal of interest in isolating, characterizing, and defining the functional mechanisms of growth modulatory factors because of their potential use in the diagnosis, prognosis, and treatment of cancer.

Moreover, acquiring knowledge of these factors will aid in the understanding of the basic mechanisms behind normal growth control and the loss thereof in cancer cells.

### 3. SUMMARY OF THE INVENTION

5       The present invention is directed to epithelins, a novel family of low molecular weight, cysteine-rich proteins exhibiting bifunctional growth regulatory activities, to the use of epithelins in the diagnosis and treatment of human diseases, and to methods for the  
10       production of biologically active epithelins. Two members of the epithelin family, epithelin 1 and epithelin 2, have been identified and purified to apparent homogeneity, enabling applicants to determine the primary structures, physical properties and functional characteristics of these  
15       novel growth modulators. Several other members of the epithelin family have been identified by cDNA cloning. Epithelin 1 is a polypeptide comprising 56 amino acids, while epithelin 2 comprises 57 amino acids. Structurally, epithelin 1 and epithelin 2 share 47% homology at the amino  
20       acid level and each contains 12 identically positioned cysteine residues.

      Epithelins may be produced by isolation and purification from natural sources, by chemical synthesis, or by recombinant DNA technology. In a particular  
25       embodiment of the invention, described more fully by way of example herein (Section 6, infra), epithelin 1 and epithelin 2 are isolated from rat kidney tissue and subsequently purified to apparent homogeneity using a combination of gel permeation and reversed phase high performance liquid  
30       chromatography (HPLC). As further described in Section 6, infra, applicants have thoroughly characterized the purified epithelins I and II with respect to their structural, physical, chemical, and functional  
35       characteristics.

#### 4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Preparative gel permeation HPLC of crude extract.

FIG. 2. Preparative reversed phase HPLC of pooled fractions 25-28 from 28 runs of FIG. 1.

FIG. 3. Semi-preparative reversed phase HPLC of pooled fractions 55-59 from FIG. 2.

FIG. 4. Analytical reversed phase HPLC of pool 1b from previous run.

FIG. 5. Analytical reversed phase HPLC of pool 2b from FIG. 3.

FIG. 6. Analytical gel permeation chromatography of the concentrated fractions 51(A) and 52(B) from FIG. 4.

FIG. 7. Analytical gel permeation chromatography of the concentrated fractions 44(A) and 45(B) from FIG. 5.

FIG. 8. Semi-preparative reversed phase HPLC of fractions 50-54 from FIG. 2.

FIG. 9. Analytical reversed phase HPLC of fractions 18-23 from previous run.

FIG. 10. Analytical gel permeation chromatography of fractions from FIG. 9. Chromatography was performed as described in Section 6.1., infra. (A) HPLC of concentrated fraction 36; (B) HPLC of concentrated fraction 37; (C) HPLC of concentrated fraction 38; (D) rechromatography of pooled fractions 48 and 49 from A-C, then concentrated.

FIG. 11. Tricine-SDS-PAGE analysis of epithelin 1 and epithelin 2. An 18% minigel (0.75 mm x 10 cm x 7 cm) was run at room temperature at a constant voltage of 90 volts for 4.5 hr in a Bio-Rad mini-protein II electrophoresis apparatus. Dried samples were suspended in 10 $\mu$ l sample buffer (50 mM Tris pH 6.8, 12% glycerol (w/v), 4% SDS, 4% mercaptoethanol (v/v) and 0.01% serva blue G.) incubated at 95°C for five minutes and then applied on the gel. The

molecular weight markers were five polypeptides from the cleavage of the horse heart myoglobin by cyanogen bromide (Sigma Chem. Co.). (A) epithelin 1; (B) epithelin 2.

FIG. 12. Amino acid sequences and alignment of epithelin 1 and epithelin 2 purified from rat kidney. The standard single letter code for amino acids is used:  
5 Alanine (A); Arginine (R); Asparagine (N); Aspartic acid (D); Cysteine (C); Glutamine (Q); Glutamic acid (E); Glycine (G); Histidine (H); Isoleucine (I); Leucine (L); Lysine (K); Methionine (M); Phenylalanine (F); Proline (P);  
10 Serine (S); Threonine (T); Tryptophan (W); Tyrosine (Y); and Valine (V). The peptide sequences used to design oligonucleotide primers and probes are underlined.

FIG. 13. Hydropathy analysis of epithelin 1 and 2 (Kyte and Doolittle). —, epithelin 1; ····, epithelin 2.  
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FIG. 14. Dose response curve of epithelin 1 and epithelin 2 on the inhibition of  $^{125}\text{I}$ -deoxyuridine incorporation into DNA of A431 cells. ●, epithelin 1; o, epithelin 2.

FIG. 15. (A) Effect of epithelin 1 and epithelin 2 on the stimulation of  $^{125}\text{I}$ -deoxyuridine incorporation into DNA of the murine keratinocyte cell line Balb/MK. 2,000-4,000 cells were plated per well in 96 well plates in low-calcium medium containing 5% dialyzed FBS. Then GSA assays were  
25 performed as described in Section 6.2., infra. ●, epithelin 1; o, epithelin 2. (B) Effect of various concentrations of epithelin 2 on epithelin 1 (20 ng/ml) elicited incorporation of  $^{125}\text{I}$ -deoxyuridine into DNA of Balb/MK cells.

FIG. 16. (A) Effect of epithelin 1 and epidermal growth factor (EGF) on the growth of Balb/MK cells. Assays were performed as described in Section 6.2., infra. ●,  
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epithelin 1; o, EGF (B) Effect of various concentrations of epithelin 2 on the epithelin 1 (20 ng/ml) induced growth of murine keratinocytes.

5 FIG. 17. Effect of epithelin 1 and 2 on NRK-SA6 cell colony formation in soft agar in the presence of TFG $\beta$  (1 ng/ml). The colony formation assay used is described in Section 6.2.3, *infra*. Solid bars, epithelin 1; stippled bar, epidermal growth factor; open bar, epithelin 2; hatched bar, 20 ng/ml epithelin 1 plus 500 ng/ml epithelin 2.

10 FIG. 18. Rat epithelin precursor cDNA and deduced amino acid sequences.

FIG. 19. Dot matrix alignment of the 589 amino acid rat epithelin precursor compared against itself. Each point represents a stretch of five out of ten identical residues.

15 FIG. 20. (A) Composite secondary structure analysis of rat epithelin precursor. (B) Hydropathy of rat epithelin precursor.

20 FIG. 21. (A) Protein sequence comparison between the human, rat, and mouse epithelin precursor deduced from cDNA clones. Sequences are displayed using the single-letter code with identical residues denoted with dots. Gaps were introduced for optimal alignment and are shown by a dash. The predicted rat epithelin signal sequence is underlined and the seven cysteine-rich motifs are boxed. Each sequence represents a consensus based on cDNA and PCR clones isolated from human, rat, or mouse kidney RNA. Arrows mark the boundaries of a 234 bp exon, a region absent in one rat cDNA clones. (B) Amino acid sequences of rat, mouse, and human epithelins. (C) Consensus cysteine motif conserved among the epithelins. (D) Alignment of the C-terminal domain (amino acids 254-315) of a tomato (Lycopersicon esculentum) thiol protease with epithelin 1 and 2.

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FIG. 22. Nucleotide and deduced amino acid sequence of the human epithelin precursor. Sequences are based on PCR clones from human kidney medulla RNA.

FIG. 23. Nucleotide and deduced amino sequence of the mouse epithelin precursor. PCR clones were obtained  
5 from adult mouse kidney RNA.

FIG. 24. Nucleotide and deduced amino acid sequence of the bovine epithelin precursor (partial sequence). Sequences are based on PCR clones from bovine  
10 testis RNA.

FIG. 25. Nucleotide and deduced amino acid sequence of the chicken epithelin precursor (partial sequence). Sequences are based on PCR clones from chicken  
oviduct RNA.

FIG. 26. Expression of recombinant epithelin in COS  
15 cells. (A)  $^{35}\text{S}$ -cysteine labeled supernatants from COS cells transfected with the following CDM8-based expression constructs: lane 1, crEPN1.6 containing the complete rat epithelin coding region; lane 2, crEPN1.4, containing a rat epithelin cDNA isoform lacking a 234 bp exon; lane 3,  
20 mock-transfected control. (B)  $^{35}\text{S}$ -cysteine labeled supernatants from COS cells transfected with the following CDM8 expression constructs: lane 1, c $\beta$ rEPN1, containing a simian TGF- $\beta$ 1 signal sequence preceding the coding region of mature rat epithelin 1; lane 2, c $\beta$ rEPN2, a similar  
25 plasmid based on rat epithelin 2.

FIG. 27. Dendrogram representation of a cluster analysis between the epithelin cysteine-rich motifs from rat, mouse, and human sources. Below is a diagram of the  
30 epithelin precursor showing the position of the 7 motifs within the precursor. The 28 cysteine-rich motifs were aligned on PCGENE (Intelligenetics, Inc. Mountain View, CA) using the CLUSTAL multiple alignment program. The pairwise similarity scores were transformed into a difference matrix

which was analyzed using the Ward's method of cluster analysis (SPSS/PC+, Chicago, IL). This method uses squared Eclidean distances to place branch points.

## 5. DETAILED DESCRIPTION OF THE INVENTION

5           The present invention is directed to a novel family of growth regulatory proteins termed "epithelins". The epithelins appear to comprise several distinct members sharing significant structural homology. Two members of the epithelin family, epithelin 1 and epithelin 2, have  
10       been purified from natural sources, and cDNAs encoding these and several other members of the epithelin family have been isolated from rat, human, bovine, murine and chicken, among other cell sources.

15           More particularly, the invention is directed to each and every member of the epithelin family, epithelin derivatives and analogues, epithelin-encoding nucleic acid molecules (e.g., cDNAs, genomic DNAs, RNAs, anti-sense RNAs, etc.), traditional and recombinant DNA based methods  
20       for the production of epithelins, recombinant epithelin expression vectors, and diagnostic and/or therapeutic uses of mature and precursor epithelins, epithelin-encoding nucleic acid molecules, anti-epithelin antibodies and  
      epithelin receptor(s).

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### 5.1. PRODUCTION OF EPITHELINS

      The individual epithelins may be produced by several general approaches, including isolation from natural sources, solid phase peptide synthesis, and  
30       recombinant DNA technology.

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#### 5.1.1. ISOLATION AND PURIFICATION OF EPITHELINS FROM NATURAL CELL SOURCES

Applicants' DNA cloning efforts have revealed that messenger RNAs encoding the various epithelins are expressed in a number of different cell types representing a broad species range. Therefore, applicants anticipate that the individual members of the epithelin family may be isolated from a wide variety of organs, tissues, and/or other cell sources. The epithelins may be separated from each other and purified from such cell sources by using various separation and purification techniques known in the art, including but not limited to chromatographic techniques (e.g., reversed phase liquid, gel permeation, liquid exchange, ion exchange, size exclusion, and affinity chromatography), centrifugation, electrophoretic procedures, differential solubility, etc.

In a specific embodiment of the invention, described more fully by way of example in Section 6., infra, two members of the epithelin family (epithelins 1 and 2) are isolated from rat kidney tissue and subsequently purified to apparent homogeneity using, inter alia, a combination of gel permeation and reversed phase high performance liquid chromatographies (HPLC). Epithelins 1 and 2 purified in this manner are single chain polypeptides comprising 56 and 57 amino acid residues, respectively, and share significant structural characteristics. Functionally, epithelin 1 appears to be a true bifunctional growth modulator, capable of stimulating and inhibiting cell growth. Epithelin 2 appears functionally distinct inasmuch as it specifically antagonizes the cell growth stimulatory activity induced by epithelin 1. Like epithelin 1, though generally to a lesser degree, epithelin 2 is also capable of inhibiting cell growth. The functional, structural, physical and other properties of epithelin 1 and epithelin 2 have been determined and are

described in Section 6.4., infra. The six-step method of preparing purified epithelin 1 and epithelin 2 described in Section 6., infra, and/or modifications thereof, may also be used to isolate other members of the epithelin family.

5                   5.1.2. CHEMICAL SYNTHESIS OF EPITHELINS

The individual members of the epithelin family may be produced using chemical methods to synthesize the corresponding amino acid sequences in whole or in part. For example, epithelins may be synthesized by solid phase techniques (Stewart and Young, Solid Phase Peptide Synthesis, 2nd edition, 1984). Purification and/or refolding into biologically active conformations of epithelins synthesized in this manner may be accomplished by various techniques known in the art. The amino acid compositions of the synthesized epithelins may be confirmed by amino acid analysis.

                  5.1.3. SYNTHESIS OF EPITHELINS USING RECOMBINANT DNA TECHNOLOGY

20                   Biologically active mature and precursor epithelins may be produced by the expression of epithelin-encoding DNAs in a recombinant host cell system. General techniques for the isolation of gene sequences, the construction of vectors capable of directing the synthesis of encoded proteins, and the expression and/or secretion of biologically active recombinant proteins are well known in the art.

                  Production of an epithelin using recombinant DNA technology may be divided into a four-step process for the purposes of description: (1) isolation or generation of the coding sequence (gene) for a precursor or mature form of the epithelin; (2) construction of an expression vector capable of directing the synthesis of the desired epithelin; (3) transfection or transformation of

appropriate host cells capable of replicating and expressing the epithelin gene and/or processing the gene product to produce the desired epithelin; and (4) identification and purification of the desired epithelin product.

5           The cloning of a rat epithelin precursor, its expression, and the expression of mature rat epithelin 1 and 2 are described by the examples presented in Section 7., et seq, infra.

10           5.1.3.1. ISOLATION OR GENERATION OF EPITHELIN GENES

          The nucleotide coding sequences of the various individual epithelins, or functional equivalents thereof, may be used to construct recombinant expression vectors which will direct the expression of the desired epithelin  
15           product. Epithelin-encoding nucleotide sequences may be obtained from a variety of cell sources which produce epithelin-like activities or which express epithelin-encoding mRNA. Applicants have identified a number of suitable human and murine tissue sources in this regard,  
20           including but not limited to placenta, colon, kidney, testes, adrenal, breast, ovary, duodenum, thymus, and lung tissues.

          Epithelin coding sequences may be obtained by cDNA cloning from RNA isolated and purified from such cell  
25           sources or by genomic cloning. Either cDNA or genomic libraries of clones may be prepared using techniques well known in the art and may be screened for particular epithelin-encoding DNAs with nucleotide probes designed from the known amino acid sequence of epithelin 1 or  
30           epithelin 2 and/or which are substantially complementary to any portion of the epithelin gene. Full length clones, i.e., those containing the entire coding region of the precursor or mature epithelin desired may be selected for  
35           constructing expression vectors.

Alternatively, epithelin-encoding DNAs may be synthesized in whole or in part by chemical synthesis using techniques standard in the art.

Due to the inherent degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence may be used in the practice of the methods of the invention. Such alterations of epithelin nucleotide sequences include deletions, additions or substitutions of different nucleotides resulting in a sequence that encodes the same or a functionally equivalent gene product. The gene product may contain deletions, additions or substitutions of amino acid residues within the sequence which result in silent changes thus producing a bioactive product. Such amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; amino acids with uncharged polar head groups or nonpolar head groups having similar hydrophilicity values include the following: leucine, isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine; phenylalanine, tyrosine.

#### 5.1.3.2. CONSTRUCTION OF EPITHELIN EXPRESSION VECTORS

In order to express biologically active, mature or precursor forms of the various epithelins, an expression vector/host system should be chosen which provides not only for high levels of transcription and translation but for the correct processing of the gene product. This may be especially important when employing the entire coding sequence of an epithelin precursor in the expression

constructs since the mature forms of the epithelins appear to be derived from larger precursors via cellular processing events.

A variety of animal/host expression vector systems (i.e., vectors which contain the necessary elements for directing the replication, transcription and translation of epithelin coding sequences in an appropriate host cell) may be utilized equally well by the skilled artisan. These include, but are not limited to, virus expression vector/mammalian host cell systems (e.g., cytomegalovirus, vaccinia virus, adenovirus, and the like); insect virus expression vector/insect cell systems (e.g., baculovirus); or nonviral promoter expression systems derived from the genomes of mammalian cells (e.g., the mouse metallothioneine promoter). Appropriate host cells include but are not limited to mammalian cells. For example, transient expression of mammalian proteins may be achieved using a COS cell host, while stable expression may be achieved using a CHO cell host.

The expression elements of these vectors vary in their strength and specificities. Depending on the host/vector system utilized, any one of a number of suitable transcription and translation elements may be used. For instance, when cloning in mammalian cell systems, promoters isolated from the genome of mammalian cells, (e.g. mouse metallothioneine promoter) or from viruses that grow in these cells, (e.g. vaccinia virus 7.5K promoter or Moloney murine sarcoma virus long terminal repeat) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the inserted sequences.

Specific initiation signals are also required for sufficient translation of inserted protein coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire epithelin

gene including its own initiation codon and adjacent sequences are inserted into the appropriate expression vectors, no additional translational control signals may be needed. However, in cases where only a portion of the coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the epithelin coding sequences to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of transcription attenuation sequences, enhancer elements, etc.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing the epithelin gene of interest and appropriate transcriptional/translational control signals. These methods may include in vitro recombinant DNA techniques, synthetic techniques and in vivo recombinations.

For example, in cases where an adenovirus is used as an expression vector, an epithelin coding sequence may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome that is viable and capable of expressing the epithelin in infected hosts. Similarly, the vaccinia 7.5K promoter may be used.

An alternative expression system which could be used to express epithelins is an insect system. In one such system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes.



The virus grows in Spodoptera frugiperda cells. An epithelin coding sequence may be cloned into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of an epithelin coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat encoded by the polyhedrin gene). These recombinant viruses are then used to infect Spodoptera frugiperda cells in which the inserted gene is expressed.

Retroviral vectors prepared in amphotropic packaging cell lines permit high efficiency expression in numerous cell types. This method allows one to assess cell-type specific processing, regulation or function of the inserted protein coding sequence.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers, (e.g. zinc and cadmium ions for metallothionein promoters). Therefore, expression of the genetically engineered epithelins may be controlled. This is important if the protein product of the cloned foreign gene is lethal to the host cell. Furthermore, modifications (e.g. glycosylation) and processing (e.g., cleavage) of protein products are important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the expressed foreign protein.

5.1.3.3. IDENTIFICATION OF TRANSFECTANTS OR TRANSFORMANTS  
EXPRESSING EPITHELIN GENE PRODUCTS

The host cells which contain the recombinant coding sequence and which express the biologically active, mature product may be identified by at least four general approaches (a) DNA-DNA, DNA-RNA or RNA-antisense RNA hybridization; (b) the presence or absence of "marker" gene functions; (c) assessing the level of transcription as measured by the expression of epithelin mRNA transcripts in the host cell; and (d) detection of the mature gene product as measured by immunoassay and, ultimately, by its biological activities.

In the first approach, the presence of epithelin coding sequences inserted into expression vectors can be detected by DNA-DNA hybridization using probes comprising nucleotide sequences that are homologous to epithelin coding sequences.

In the second approach, the recombinant expression vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, etc.). For example, if an epithelin coding sequence is inserted within a marker gene sequence of the vector, recombinants containing that coding sequence can be identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with the epithelin sequence under the control of the same or different promoter used to control the expression of the epithelin coding sequence. Expression of the marker in response to induction or selection indicates expression of the epithelin coding sequence.

In the third approach, transcriptional activity for an epithelin coding region can be assessed by hybridization assays. For example, polyadenylated RNA can be isolated and analyzed by Northern blot using a probe homologous to the appropriate epithelin coding sequence or particular portions thereof. Alternatively, total nucleic acids of the host cell may be extracted and assayed for hybridization to such probes.

In the fourth approach, the expression of the mature protein product can be assessed immunologically, for example by Western blots, immunoassays such as radioimmunoprecipitation, enzyme-linked immunoassays and the like. The ultimate test of the success of the expression system, however, involves the detection of the biologically active epithelin gene product. Where the host cell secretes the gene product, cell free media obtained from cultured transfectant host cells is assayed for epithelin activity. Where the gene product is not secreted, cell lysates may be assayed for such activity. In either case, biological assays such as the growth inhibition and stimulation assays described herein or the like may be used.

#### 5.1.4. EPITHELIN DERIVATIVES, ANALOGS AND PEPTIDES

The production and use of derivatives, analogues, and peptides related to the epithelins are also envisioned and are within the scope of the invention. Such derivatives, analogues, and peptides which exhibit growth modulatory activity may, like the various epithelins, find applications in the diagnosis, prognosis, and treatment of a wide variety of neoplasias and other growth related diseases. Such derivatives, analogues, or peptides may have enhanced or diminished biological activities in comparison to native epithelins and/or may expand or limit epithelin growth inhibitory activity (GIA)-susceptible cell

range and still be within the scope of the invention. Similarly, the production and use of derivatives, analogues, and peptides related to epithelins which exhibit enhanced or diminished growth stimulatory activity (GSA) and/or which expand or limit the range of cells responsive to epithelin GSA may find useful applications including, but not limited to, the treatment of wounds and burns.

Epithelin-related derivatives, analogues, and peptides of the invention may be produced by a variety of means known in the art. Procedures and manipulations at the genetic and protein levels are within the scope of the invention.

At the protein level, numerous chemical modifications could be used to produce epithelin-like derivatives, analogues, or peptides by techniques known in the art, including but not limited to acetylation, formylation, oxidation, specific chemical cleavage by endopeptidases (e.g. cyanogen bromide, trypsin, chymotrypsin, V8 protease, and the like) or exopeptidases, etc.

## 5.2. ANTI-EPITHELIN ANTIBODIES

Also within the scope of the invention is the production of polyclonal and monoclonal antibodies which recognize epithelins or related proteins.

Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of epithelins. For the production of antibodies, various host animals can be immunized by injection with an epithelin, or a synthetic epithelin peptide, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels (such as aluminum hydroxide), surface active substances (such as lysolecithin), pluronic

polyols, polyanions, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

5 A monoclonal antibody to an epitope of an epithelin can be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975, Nature 256, 495-497), and the  
10 more recent human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72) and EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

15 Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the  $F(ab')_2$  fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of  
20 the  $F(ab')_2$  fragment, and the two Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

Antibodies to epithelins may find use in the qualitative detection of mature epithelins and their  
25 precursor and subcomponent forms, in the affinity purification of epithelin proteins, and in the elucidation of epithelin biosynthesis, metabolism and function. Antibodies to epithelins may also be useful as diagnostic and therapeutic agents.  
30

### 5.3. BIOLOGICAL PROFILE OF THE EPITHELINS

Applicants' initial cDNA cloning efforts indicate that the epithelin family of growth modulatory  
35 proteins is comprised of several structurally similar

members. Moreover, it appears that epithelin-encoding mRNA is expressed in several tissue types over a broad range of chordates. Applicants' initial data regarding the structure of epithelin genes from these various chordate sources suggests that the epithelin gene has been in place and has remained remarkably constant for at least 250 million years. The various epithelins appear to be single chain low molecular weight proteins. None of the sequences obtained for the epithelins are significantly homologous to any previously known protein. Interestingly, several of the epithelins contain a region homologous with the active site of phospholipase A2.

Epithelin 1 and epithelin 2 purified from rat kidney tissue are proteins of 56 and 57 amino acids, respectively, sharing 47% amino acid sequence homology and 12 identically positioned cysteine residues. Thus, about one-fifth of the amino acids of both epithelins are cysteines. The positioning of these 12 cysteine residues suggests that, through the formation of disulfide linkages between them, the pattern directs the formation of a "cysteine cage" at the tertiary structural level, perhaps not unlike zinc finger structures. Applicants are not aware of any other protein having this particular, or a closely related, cysteine pattern. This unique cysteine pattern is observed not only in the primary structures of purified epithelins 1 and 2, but also in the structures deduced from the sequences of several epithelin-encoding cDNAs and PCR clones. It appears that this unique cysteine pattern is therefore a distinguishing characteristic of the epithelin family members, and likely plays an important functional role. In addition, some 15 other amino acids are conserved between these two purified epithelins.

A cDNA encoding the complete rat epithelin precursor has been isolated and its nucleotide and deduced amino acid sequences determined, as shown in FIG. 18. The

rat epithelin precursor comprises a polypeptide of 589 amino acid residues, the amino-terminal 17 residues of which comprise its signal peptide. It appears that at least seven complete and distinct epithelin species are encoded within the rat epithelin precursor gene. The high  
5 level of sequence homology between the epithelins encoded by the rat epithelin precursor cDNA can be visualized by the 7.5-fold internal homology generated upon comparison of the rat epithelin precursor sequence to itself, as illustrated in FIG. 19. Composite secondary structure and  
10 hydropathy analyses of the rat epithelin precursor are shown in FIG. 20A and FIG. 20B, respectively. Amino acid sequence alignments between the rat, mouse and human epithelin precursors are shown in FIG. 21A.

Applicants have also isolated the complete  
15 human, mouse and rat epithelin precursor DNA sequences. A composite alignment of these epithelin sequences is illustrated in FIG. 21A. In addition, a number of PCR clones encoding various epithelins of bovine and avian origin have been obtained (FIG. 24, 25). Analysis of the  
20 various sequences indicates that the epithelins share, to somewhat different degrees, a distinct structural characteristic defined by the highly conserved cysteine motif (consensus) shown in FIG. 21C. While this consensus sequence appears to be generally conserved among the  
25 epithelins, a cysteine core motif of "CCx<sub>8</sub>CCx<sub>6</sub>CCx<sub>5</sub>CC" is almost completely conserved in all epithelin species examined to date, regardless of origin. Exceptions include the first full repeat of the rat epithelin precursor where Cysteine to serine substitutions are present and the motif  
30 is "SCx<sub>9</sub>CCx<sub>6</sub>SCx<sub>5</sub>CC". Furthermore, a histidine residue at the 25th position of the core motif also appears to be conserved among the epithelin species. Applicants believe that the core motif is a unique characteristic of all  
35 epithelins and, accordingly, intend that the present

invention encompass all proteins having this structural feature. FIG. 21B shows the amino acid sequence of human, rat and mouse mature epithelins 1-7 aligned. The sequence represented by FIG. 21C is completely conserved between epithelins 2-7 in these three species. The epithelin 1  
5 sequences diverge from this sequence only slightly.

Applicants have examined RNA from a large number of human and murine tissues and cell lines for the presence of epithelin transcripts, the results of which are  
10 summarized in TABLE I, below.

15

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30

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TABLE I

5      DISTRIBUTION OF EPITHELIN TRANSCRIPT EXPRESSION IN VARIOUS  
HUMAN AND MURINE TISSUES AND CELLS

	<u>HUMAN</u> <u>Strong +</u>	<u>Weak +</u>	<u>Negative</u> (using Rat Probe)
10	Placenta Colon Kidney Medulla Kidney Cortex Testis Adrenal Breast	Ovary Duodenum Thymus Lung Kidney	Brain Epidermis Liver Pituitary Amnion Bone Marrow Cerebellum
15	CRL 7386 Caki-1 CRL 1550 Caki-2 HUF CCL 137	HB1100 HEPM CRO 1572 HTB132 1477 HSB2 U937 HTB131 BT474 HTB36	MCF-7 HTB27 T47D CEMA-1  Breast Ca Wilm's Ca
20			
	<u>MOUSE</u> <u>Strong +</u>	<u>Weak +</u>	<u>Negative</u>
25	Fetal Intestine Placenta Kidney  Brain (Cortex)	Heart Ovary Thymus Pancreas Cerebellum Lung	NONE
30		Embryo d15 Embryo D6 Liver Colon Duodenum Skeletal Muscle  CCL51 CCL51 (TPA)	

The biological characteristics of purified epithelin 1 and epithelin 2 are described in some detail in Section 6.4, infra. Both proteins are stable after treatment with 1M acetic acid, 1M ammonium hydroxide, 6M urea, 10mM sodium metaperiodate, and heating at 56°C for 30 minutes. The bioactivities of both proteins are sensitive to, inter alia, proteolytic enzymes and reducing agents. It is clear that at least some disulfide linkages in the epithelin structure are essential for bioactivity. It does not appear that oligosaccharides and/or lipid moieties are essential for the activity of either epithelin 1 or 2.

Epithelin 1 and epithelin 2 exhibit growth inhibitory activity on, inter alia, human epidermoid carcinoma cells. Applicants' data suggests, however, that epithelin 1 is a more potent inhibitor of cell growth. For example, the calculated specific activity of purified epithelin 1 is nearly ten times that of epithelin 2, and epithelin 1 appears to be some 36 times more potent than epithelin 2 in inhibiting the growth of A431 cells. Moreover, in at least one cell line tested, a human colon carcinoma cell line, epithelin 2 was incapable of triggering the inhibitory effect observed with epithelin 1.

Of additional interest is the functional divergence between epithelin 1 and epithelin 2 with respect to cell growth stimulatory bioactivity. Applicants have demonstrated that epithelin 1, in addition to its growth inhibitory bioactivity, is a potent stimulator of cell growth on several cell lines, leading applicants to conclude that epithelin 1 is a true bifunctional growth modulator. In contrast, applicants' data suggests that epithelin 2 is incapable of triggering a growth stimulatory effect, at least on the cell lines tested.

Perhaps most interesting of all is applicants' further discovery that epithelin 2 specifically antagonizes the stimulatory activities exerted by epithelin 1.

Although the functional interrelationship among these and/or other epithelins is not understood at the present time, it is possible that epithelin 2 may function as a control on the stimulatory activity induced by epithelin 1. Applicants speculate that an agonist/antagonist functional relationship among epithelin 1 and epithelin 2, and/or among other members of the epithelin family, may control or otherwise influence the balance between normal and unrestrained cell growth and development. In this regard, cellular homeostasis may be altered or destroyed by the loss of a critical function provided by an epithelin or epithelins involved in such an interrelationship resulting, for example, in unrestrained cell proliferation. Through the therapeutic use of epithelins, anti-epithelin antibodies, and/or epithelin receptors, cellular homeostasis may be modulated and/or restored.

The ability to express individual motifs from the epithelin precursor will assist in determining whether differential processing releases other active molecules. In order to select candidate effectors or blockers, the sequences of the cysteine-rich motifs shown in FIG. 21A were aligned and cluster analyzed. The results are represented by the dendrogram in FIG. 27. In all cases, the least dissimilar motif is found at the same position within the precursor of the other 2 species. Based on these findings, it is proposed that the primordial epithelin gene underwent a seven-fold replication prior to the divergence of rodents and humans. This analysis also shows that the fifth repeat of the epithelin precursor is most similar to epithelin 1, and is a candidate for having growth stimulatory activity. In addition, the second repeat is most similar to epithelin 2, and is a candidate antagonist of the mitogenic effects of epithelin 1.

The epithelin gene has several intriguing features. The gene's ubiquitous expression suggests it plays a role in the maintenance of normal epithelial cell growth, in contrast to previously described molecules that have a more restricted distribution. The highly repetitive and cysteine-rich structure of the epithelin precursor defines a novel and evolutionarily conserved motif. Furthermore, at least two of these motifs can be proteolytically processed into active growth regulators. This configuration is similar to that of proopiomelanocortin (POMC), a prohormone that is processed in a tissue-specific manner to release a variety of bioactive peptides. (Smith and Funder, 1988, Endocr. Rev. 9:159-79).

The opposing activities of epithelin 1 and 2 on the growth of epithelial cells is reminiscent of other systems where naturally occurring, structurally related molecules act as antagonists or suppressors of the parent molecule. Examples of this include; IL-1ra, an interleukin-1 receptor antagonist (Hannum et al., 1990, Nature 343:336-40; Eisenberg et al., 1990, Nature 343:341-46); Krev-1 (Kitayama et al., 1989, Cell 56:77-84), a protein that suppresses ras induced transformation; and inhibin (Ling et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 82:7217-21), a gonadal protein that opposes the biological effects of activin (Ling et al., 1986, Nature 321:779-82). Further studies to identify cellular receptors for epithelin 1 and 2 are needed to define how these molecules mediate their opposing signals. The finding that both activities are products of the same transcript is provocative. Conceivably, tissue-, spatial-, or temporally-specific processing might provide a unique means of regulating epithelial homeostasis.

5.4. USES OF THE EPITHELINS, EPITHELIN-ENCODING  
NUCLEIC ACID MOLECULES, ANTI-EPITHELIN  
ANTIBODIES AND EPITHELIN RECEPTORS

Applicants envision a wide variety of uses for the compositions of the present invention, including  
5 diagnostic and/or therapeutic uses of the epithelin proteins, epithelin analogues and derivatives, epithelin-encoding nucleic acid molecules, anti-epithelin antibodies and epithelin receptors.

10 5.4.1. EPITHELIN PROTEINS

Epithelin proteins, analogues and derivatives, as well as compositions containing them, may be used alone or in combination with each other and/or with other biologically active growth factors, inhibitors, or  
15 immunomodulatory agents to regulate the growth and/or development of chordate cells in vivo and in vitro.

Different epithelins and epithelin compositions may be used to achieve different therapeutic objectives. In particular, given the observed functional diversity  
20 between epithelin 1 and epithelin 2, applicants envision that these two epithelins may be used for different purposes. However, notwithstanding their functional differences, both epithelin 1 and epithelin 2 may be useful as anti-tumor agents since they both demonstrate the  
25 ability to inhibit the growth of neoplastic cells, although applicants' initial data suggests that epithelin 1 may be a more powerful and/or effective tumor inhibitor. The particular combination of epithelins and/or other factors used will depend on the type of target cells involved as  
30 well as the particular objective(s) desired.

For in vivo use, the subject compositions may be administered in a variety of ways, including but not limited to, injection, infusion, topically, parenterally, etc. Administration may be in any physiologically

acceptable carrier, including phosphate buffered saline, saline, sterilized water, etc. Epithelins and related molecules may also be encapsulated in liposomes and may be conjugated to antibodies which recognize and bind to tumor or cell specific antigens, thereby providing a means for  
5 "targeting" the compositions of the invention.

The epithelins may be useful in vivo for inducing terminal differentiation in tumor cells. Such cells have diverted from the ordinary course of cell differentiation characteristic of normal cells and are capable of continued  
10 proliferation. Normal cells, in contrast, differentiate into cells which are incapable, under most circumstances, of further cell division. Thus, the ability of the epithelins to reactivate normal cellular differentiation in tumors and, ultimately, to arrest continued tumor growth  
15 may find valuable use in tumor therapy regimens.

Epithelins and related derivatives, analogues, and peptides thereof may be used alone or with at least one other anti-proliferative compound, including, for example, an interferon, TFG- $\beta$ , tumor necrosis factors, etc., in the  
20 treatment of neoplastic and other growth related diseases. Carcinomas may also be treated by inducing production of epithelins in the carcinoma cells.

The compounds of the invention may be used in vitro to inhibit the growth of cells or cell lines  
25 sensitive to epithelins as distinguished from cells which are not sensitive. In this way, heterogeneous mixtures or cell lines can be freed of undesirable cells, where the undesirable cells are sensitive to epithelin growth inhibitory activity. For example, the compounds of the  
30 invention may be used in vitro to eliminate malignant cells from marrow for autologous marrow transplants, and to eliminate or inhibit the proliferation of malignant cells in blood prior to reinfusion.

35

The most effective concentration of epithelins for inhibiting proliferation of a given cell may be determined by adding various concentrations of epithelins to the tumor cell of interest and monitoring the amount of inhibition of cell proliferation. The most effective  
5 concentration of individual inducers and/or combinations of inducers may be determined by monitoring the production of epithelins in the carcinoma cells.

Stimulation of cell growth can be induced by epithelin 1, epithelin 1-like molecules, and, perhaps, by  
10 other members of the epithelin family. A wide range of therapeutic applications based on this epithelin bioactivity are envisioned, including but not limited to wound healing and tissue remodeling. Moreover, antibodies capable of neutralizing the epithelin 1-inhibiting activity  
15 of epithelin 2 may also be useful in promoting wound healing and tissue remodeling, with or without the coadministration of epithelin 1 or epithelin 1-like molecules.

The compositions of the present invention may  
20 also find use in the treatment of human skin diseases involving the proliferation of normal cells, such as psoriasis. Although the pathogenesis of psoriasis is not known, the disease involves rapid epithelial cell proliferation and turnover. The accompanying rapid  
25 turnover of keratinocytes alters keratinization, resulting in thickened epidermis and scales characteristic of the disease. Since epithelin 1 stimulates the growth and proliferation of keratinocytes, effective therapy inhibiting this epithelin 1-induced activity may impede the  
30 onset and development of the disease. Therefore, compositions capable of inhibiting what may be endogenous or abnormally high levels of epithelin 1 in psoriasis patients may be effective in curing the disease.  
35 Applicants have demonstrated that epithelin 2 specifically

inhibits the epithelin-I-induced stimulation of keratinocytes. In this regard, epithelin 2-containing compositions may be particularly useful in the treatment of psoriasis. Similarly, antibodies capable of neutralizing epithelin 1 stimulatory activity may be used to inhibit epithelin 1 activity.

Applicants also envision the use of epithelins and epithelin-like molecules for other therapeutic purposes, including but not limited to the modulation of angiogenesis, renal generation, bone resorption, immune responses, synaptic and neuronal effector functions, the arachidonic cascade, and gonadal and reproductive functions.

A number of diagnostic uses of epithelins and related molecules are envisioned. In the practice of the invention, the subject polypeptides may be joined to a label, such as a radioisotope, enzyme, fluorescer, chemiluminescer, enzyme fragment, particle, etc. Such compounds may be used to titrate the number of epithelin receptors on a cell. Identification of epithelin receptors is an indication of potential responsiveness of the cell to the biological effects of epithelins and related molecules. Epithelins, epithelin-related molecules, and/or antibodies thereto may be used in competitive assays for detection of epithelins in media, particularly in physiological media. A wide variety of diagnostic assays known in the art may be used.

The presence and levels of epithelins in body fluids and tissues may directly or inversely relate to the presence and pervasiveness of certain cancers and other growth related diseases. Assays which can detect and/or quantify epithelins may find use in diagnosis and prognosis of growth related diseases.



In addition, malignant cells expressing epithelin receptors may be detected by using labeled epithelins or epithelin-related molecules in a receptor binding assay, or by the use of antibodies to the epithelin receptor itself. Cells may be distinguished in accordance with the presence  
5 and density of epithelin receptors, thereby providing a means for predicting the susceptibility of such cells to the biological activities of epithelins.

#### 5.4.2. EPITHELIN-ENCODING NUCLEIC ACID MOLECULES

10 Epithelin-encoding nucleic acid molecules or fragments thereof may be used as probes to detect and quantify mRNAs encoding epithelins. Assays which utilize nucleic acid probes to detect sequences comprising all or  
15 part of a known gene sequence are well known in the art. Epithelin mRNA levels may indicate emerging and/or existing neoplasia as well as the onset and/or progression of other human diseases including but not limited to psoriasis. Therefore, assays which can detect and quantify epithelin  
20 mRNA may provide a valuable diagnostic tool.

Anti-sense epithelin RNA molecules may be useful therapeutically to inhibit the translation of epithelin-encoding mRNAs where the therapeutic objective involves a desire to eliminate the presence of a given epithelin. Epithelin 1 anti-sense RNA, for example, could be useful as  
25 an epithelin 1 antagonizing agent in the treatment of diseases for which epithelin 1 is a causative agent. Additionally, epithelin anti-sense RNAs may be useful in elucidating epithelin functional mechanisms.

Epithelin-encoding nucleic acid molecules may be  
30 used for the production of recombinant epithelin proteins and related molecules, as separately discussed in Section 5.1.3., supra.

6. EXAMPLE: PREPARATION OF PURIFIED EPITHELIN 1  
AND EPITHELIN 2 FROM RAT KIDNEY

6.1. PURIFICATION PROCEDURES

5

6.1.1. ACID ETHANOL EXTRACTION

Rat kidneys were obtained from Pel-Freeze (Rogers, Arkansas). Frozen rat kidneys (430 g wet weight) were suspended in 2370 ml of extraction buffer consisting of 2348 ml ethanol (98%), 19 ml of concentrated HCl, 81.5 mg phenylmethyl-sulfonyl fluoride and 2.8 ml of aprotonin (23 TIU/ml from bovine lung; Sigma Chemical Co.). The tissue was allowed to thaw at 4°C for 4-6 hours and the mixture was homogenized in a Waring blender. The mixture was stirred at 4°C overnight, centrifuged at 9,000 rpm in a Sorvall GS-3 rotor for 40 minutes and the supernatant carefully removed (2,200 ml). Chloroform (2,200 ml) and 220 ml of acidified water (375 ml water + 7.5 ml of concentrated HCl) was added to the supernatant, the mixture stirred vigorously for approximately one hour, and allowed to stand at room temperature to separate into two phases. The upper aqueous phase was carefully removed and dialyzed against 17 liters of 0.1 M acetic acid at 4°C in No. 3 Spectropore dialysis tubing (molecular weight cut off approximately 3,000). The dialysis buffer was changed three times over a two-day period. The retentate was lyophilized and the lyophilized material (4.55 g), termed "crude extract", was stored at -20°C until further use.

30

6.1.2. PREPARATIVE GEL PERMEATION CHROMATOGRAPHY

A Bio-Sil TSK-250 column (21.5 x 600 mm) (BioRad) was attached to a high performance liquid chromatography (HPLC) system (Waters). The crude extract (25 mg/ml) was dissolved in 50% acetonitrile/water with 0.1% trifluoroacetic acid (TFA). A 3 ml aliquot of the mixture

35

was injected and elution was performed isocratically with a mobile phase of 50% acetonitrile with 0.1% TFA. The flow rate was 4 ml/min and chart speed was set at 0.25 cm/min. Six ml fractions were collected. The chromatography was performed at room temperature. An aliquot from each fraction was evaporated and assayed in triplicate for growth inhibitory activity (GIA) on A431 human epidermoid carcinoma cells as described in Section 6.2.1., infra. (FIG. 1).

The late eluting minor peak (Fractions 25-28) contained the new activities of interest. Fractions 25-28 from 57 similar runs were pooled, concentrated and lyophilized. The lyophilized material weighed 473 mg and had a total of approximately  $1.1 \times 10^5$  GIA units.

#### 6.1.3. REVERSED-PHASE HPLC OF PREPARATIVE TSK-250 FRACTIONS

The lyophilized fractions (Section 6.1.2., supra) were dissolved in 240 ml of 0.1% TFA in water; the mixture was centrifuged, and the supernatant was carefully removed. The final volume was about 250 ml. 125 ml of this mixture was isocratically injected onto a preparative Partisil 10 ODS-3 column (10 micron, 2.2 x 25 cm; Whatman) attached to a HPLC system. The flow rate was set at 4 ml/min. Once the sample had passed onto the column, the column was washed with 150 ml of 0.1% TFA in water. The linear gradient was generated between the primary solvent, 0.1% TFA in water, and the secondary solvent, acetonitrile containing 0.1% TFA. The gradient conditions were: 0 to 45% in 270 minutes and 45 to 100% in 45 minutes. 14 ml fractions were collected and aliquots of each fraction were assayed for GIA. Four broad peaks of activity were seen (FIG. 2). A second run was performed as described above. Two early eluting peaks, peak a and peak b, contained epithelin 2 and epithelin 1, respectively, and they were

further purified and characterized. The further purifications of epithelin 1 and epithelin 2 are described separately below.

5      6.1.4. FURTHER PURIFICATION OF EPITHELIN 1 BY REVERSED-PHASE AND GEL PERMEATION HPLC

            Fractions 55-59 (FIG. 2) from two runs were pooled and diluted twofold with 0.1% TFA in water. The mixture was isocratically injected onto a semi-preparative  $\mu$ -Bondapak-C18 column (7.8 x 300 mm, Waters) at a flow rate  
10 of 2 ml/min at room temperature. The linear gradient conditions between primary solvent, water with 0.1% TFA, and the secondary solvent, acetonitrile with 0.1% TFA, were 0 to 18% in 1.8 minutes, 18 to 18% in 20 minutes, 18 to 34% in 240 minutes, and 34 to 100% in 10 minutes. The flow  
15 rate was 2 ml/min throughout the gradient; 7 ml fractions were collected. Aliquots were taken and assayed for GIA. Two peaks of activity were observed eluting at acetonitrile concentrations of approximately 24% and 25%, respectively (FIG. 3).

20             Fractions 30-34 were pooled. 45 ml of 0.1% TFA in water was added to the pooled fraction. The mixture was isocratically applied onto a  $\mu$ -Bondapak-CN column (3.9 x 300 mm, Waters) at a flow rate of 1 ml/min at room temperature. The gradient conditions were 0 to 10% in 1  
25 minute, 10 to 10% in 19 minutes, 10 to 30% in 200 minutes, and 30 to 100% in 7 minutes. The flow rate was 0.5 ml/min and 1.5 ml fractions were collected. Most of the activity emerged from the column at about 21.5% acetonitrile concentration (FIG. 4).

30             Fractions 36-43 were pooled and diluted with 0.1% TFA/H<sub>2</sub>O to a final volume of 115 ml and chromatographed exactly as described for fractions 30-34, above. Most of the activity eluted from the column in two peaks, eluting  
35 at approximately 22.5% and 23.5% acetonitrile (FIG. 5).

Fractions 51 and 52 (FIG. 4) were individually concentrated, using a speed-vac concentrator (Savant), to a volume of about 70  $\mu$ l, to which an equal volume of acetonitrile containing 0.1% TFA was added. This 140  $\mu$ l sample was injected onto two Bio-Sil TSK-250 columns (7.5 x 300 mm each, Bio-Rad) arranged in tandem. The elution was performed isocratically with a mobile phase of 50% acetonitrile/H<sub>2</sub>O with 0.1% TFA at room temperature. The flow rate was 0.4 ml/min and chart speed was 0.25 cm/min; 0.4 ml fractions were collected and aliquots were assayed for GIA. The chromatographic profiles of fractions 51 and 52 are shown in FIG. 6A and FIG. 6B, respectively.

Fractions 44 and 45 (FIG. 5) were individually concentrated to 70  $\mu$ l and then subjected to gel permeation chromatography as described above. The chromatographic profiles are given in FIG. 7.

#### 6.1.5. FURTHER PURIFICATION OF EPITHELIN 2 BY REVERSED-PHASE AND GEL PERMEATION HPLC

Fractions 50-54 (FIG. 2) from two runs were pooled and diluted twofold with 0.1% TFA/H<sub>2</sub>O. The mixture was applied onto a semi-preparative  $\mu$ -Bondapak-C18 column (7.8 x 30 mm, Waters) at a flow rate of 2 ml/min. The linear gradient conditions between primary solvent, water with 0.1% TFA, and the secondary solvent, acetonitrile with 0.1% TFA, were 0 to 18% in 1.8 minutes, 18 to 18% in 20 minutes, 18 to 34% in 240 minutes, and 34 to 100% in 10 minutes. The flow rate was 2 ml/min throughout the gradient; 7 ml fractions were collected. Aliquots were taken and assayed for GIA. The chromatographic profile is shown in FIG. 8. The major peak of activity eluted at approximately 20.5% acetonitrile concentration.

Fractions 18-23 were pooled and diluted with 0.1% TFA/H<sub>2</sub>O to a final volume of 110 ml. The mixture was applied onto a  $\mu$ -Bondapak-CN column (3.9 x 300 mm, Waters)

at a flow rate of 1 ml/min at room temperature. The gradient conditions were 0 to 10% in 1 minute, 10 to 10% in 19 minutes, 10 to 30% in 200 minutes, and 30 to 100% in 7 minutes. The flow rate was 0.5 ml/min; 1.5 ml fractions were collected. The activity emerged from the column at an acetonitrile concentration of about 18% (FIG. 9).

Fractions 36-38 (FIG. 8) were individually concentrated to approximately 70  $\mu$ l, to which an equal volume of acetonitrile containing 0.1% TFA was added. This 140  $\mu$ l sample was applied onto two Bio-Sil TSK-250 columns (7.5 x 300 mm each, Bio-Rad) attached in tandem. The elution was performed isocratically with a mobile phase of 50% acetonitrile/H<sub>2</sub>O with 0.1% TFA. The flow rate was 0.4 ml/min; 0.4 ml fractions were collected and aliquots were assayed for GIA. The chromatographic profiles of fractions 36, 37 and 38 are shown in FIG. 10A, 10B and 10C, respectively.

Fractions 48 and 49 from FIG. 10A-C were pooled and concentrated to about 70  $\mu$ l and then subjected to gel permeation chromatography as described above. The rechromatographic profile is presented in FIG. 10D.

## 6.2. BIOASSAYS

### 6.2.1. CELL GROWTH INHIBITORY ACTIVITY USING <sup>125</sup>I-DEOXYURIDINE INCORPORATION INTO DNA

The cell growth inhibitory activity (GIA) assays were performed in flat-bottom 96 well plates (Falcon 3072). Human epidermoid carcinoma of vulva cells (A431) were used as test cells for GIA.  $3.5 \times 10^4$  cells in 50  $\mu$ l of test medium (DMEM supplemented with 5% heat inactivated fetal bovine serum (FBS), penicillin/streptomycin (PS) and glutamine) were placed in all wells except peripheral wells. The peripheral wells received 50  $\mu$ l PBS. Three hours later, 50  $\mu$ l of test sample in test medium was added

to each well, while control wells received only 50  $\mu$ l of test medium. Three wells were used for each concentration of test sample. Plates were incubated at 37°C for 2-3 days. Then, 100  $\mu$ l of a solution of  $^{125}$ I-iodo-2',- $^{125}$ I-deoxyuridine ( $^{125}$ I-IUdR, 4Ci/mg-0.5 mCi/ml, 2  $\mu$ l/ml in test medium) was added to each well and plates were incubated at 37°C. After 4-6 hours, the medium was aspirated from the wells, which were then washed once with 200  $\mu$ l PBS. Then, 200  $\mu$ l methanol was added to each well, plates were incubated for 10 minutes at room temperature, and the methanol was removed by aspiration. 200  $\mu$ l of 1M sodium hydroxide was added to each well, the plates were incubated for 30 minutes at 37°C. Sodium hydroxide was removed with titertek plugs (Flow Labs). The plugs were transferred into 12 x 75 mm plastic tubes and counted in a gamma counter to quantify  $^{125}$ I-IUdR incorporation.

#### 6.2.2. CELL GROWTH INHIBITORY AND STIMULATORY ACTIVITY ASSAYS USING MURINE KERATINOCYTES

Balb/MK cells were plated at  $1 \times 10^4$  cells per well in 1 ml of low calcium medium (Weissman and Aaronson, 1983, Cell 32:599-606; Carpenter and Zendeget, 1985, Anal. Biochem. 153:279-282) in 24-well Costar plates and incubated at 37°C for 4-6 hours. Then media were removed and replaced with 1 ml of medium containing various concentrations of the test compound in triplicate. The control wells received only medium without any test material. The plates were incubated at 37°C for 4 days, then medium was removed, wells were rinsed two times with 1 ml of phosphate-buffered saline, and the cells were detached with trypsin-EDTA and counted.

Balb/MK cells were also used as indicator cells in 96 well plates to assess the growth inhibitory activity (GIA) or growth stimulatory activity (GSA) of a test material using  $^{125}\text{I}$ -deoxyuridine incorporation into DNA as described in the previous section.

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### 6.2.3. SOFT AGAR COLONY ASSAY

A 0.38 ml base layer of 0.5% agar (Agar Noble; Difco Laboratories, Detroit, Michigan) in DMEM containing 10% heat inactivated FBS was added to 24 well Costar tissue culture plates. 0.38 ml of 0.3% agar containing the same medium-FBS mixture,  $6-12 \times 10^3$  test cells, and the test proteins at various concentrations were overlaid on the basal layer of agar. The plates were incubated at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$  in air. Colonies were enumerated unfixed and unstained, and the number of colonies was scored between days 7 and 10. Colonies were defined as a cluster of at least eight cells.

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### 6.3. PRIMARY STRUCTURE DETERMINATIONS

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#### 6.3.1. REDUCTION AND S-PYRIDYLETHYLATION

Protein (10-20  $\mu\text{g}$ ) was dried in a 1.5 ml microfuge polypropylene tube, suspended in 100  $\mu\text{l}$  of 3 M urea in 0.05 M Tris-HCl, pH 7.5. Then, 4  $\mu\text{l}$  of 2-mercaptoethanol was added to the mixture, the contents were mixed, flushed with nitrogen, and incubated at 25°C. After 2.5 hours, 4.5  $\mu\text{l}$  of freshly distilled 4-vinylpyridine was added to the mixture, the tube was again flushed with nitrogen and incubated for 2 hours at 25°C. The reaction mixture was acidified to pH 2.0 with 10% TFA. S-pyridylethylated protein was purified by reversed phase HPLC using a Partisil 5 ODS-3 column (4.6 x 100 mm, Whatman). The concentration of acetonitrile was increased linearly (1%/min) during 55 minutes at a flow rate of 1

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ml/min. The primary solvent was 0.1% TFA/H<sub>2</sub>O. S-pyridylethylated-epithelin 1 (SPE-Epithelin 1) and SPE-Epithelin 2 eluted at about 25% and 23% of acetonitrile, respectively, approximately 2-3% higher acetonitrile concentrations than the untreated epithelins.

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#### 6.3.2. ENZYMATIC CLEAVAGE OF SPE-EPITHELIN 1 AND SPE-EPITHELIN 2

Cleavage with endopeptidase Lys-C and TPCK-trypsin was performed in 60 µl of 0.1M Tris-acetic acid buffer, pH 8.0 at 25°C for 16 hours. The enzyme/substrate ratio was 1 to 5 (w/w). Endopeptidase-Arg and *S. aureus* V8 protease digestions were done in 80 µl of 0.05M Tris-HCl, pH 8.0, 0.1M ammonium-bicarbonate at 37°C for 16 hours. The enzyme/substrate ratio was again 1 to 5.

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#### 6.3.3. PEPTIDE ISOLATION

Peptides were separated on a reversed phase HPLC C18 column (4.6 x 100 mm, Whatman) attached to a HPLC system (Waters). Acidified sample (pH 2.0) was applied onto a column equilibrated with 0.1% TFA (primary solvent) at a flow rate of 1 ml/min and the column was further washed with about 15 ml of 0.1% TFA. Linear gradients were used between the primary solvent and the secondary solvent (acetonitrile with 0.1% TFA). The gradient conditions were 0 to 50% in 125 minutes at a flow rate of 0.5 ml/min.

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#### 6.3.4. AMINO ACID ANALYSIS

Dried samples were hydrolyzed with constant boiling HCl (5.7M, Pierce) containing 1% (v/v) phenol under reduced pressure in a Teflon-sealed glass hydrolysis bulb (Pierce) at 105°C for 16 hr. The hydrolysates were dried in a Speed Vac concentrator (Savant Instruments) and derivitized with phenylisothiocyanate for 20 minutes at room temperature. Phenylthiocarbamyl amino acid

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derivatives were analyzed by reversed phase HPLC on a Octadecyl column (4.5 x 250 mm, IBM). The linear gradient was performed between primary solvent 0.15M sodium acetate pH 6.4, 0.05% triethylamine titrated to pH 6.4 with acetic acid and the secondary solvent 60% acetonitrile at a flow rate of 1 ml/min at 38°C.

#### 6.3.5. AMINO ACID SEQUENCE DETERMINATION

Peptide sequences were determined with an Applied Biosystem model 475 gas phase sequencer as described (Hewick et al., 1981, J. Biol. Chem. 256:7990-7997). Three precycles of Edman degradation were performed prior to sample application for each run. 25% TFA was used to convert the Triazoline derivatives to phenylthiohydantoin amino acids. Identification of phenylthiohydantoin amino acids was carried out, on-line, on a Model 120A PTH analyzer (Applied Biosystem) as described (Hunkapiller and Hood, 1983, Science 219:650-659).

#### 6.3.6. TRICINE-SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

Proteins were analyzed on tricine/sodium dodecyl sulfate/polyacrylamide slab gels (normal or mini Bio-Rad system) by the method of Schager and Gebhard, 1987, Biochem. 166:368-379. Proteins were detected by silver staining (Wray et al., 1981, Anal. Biochem. 118:197-203).

#### 6.4. CHARACTERISTICS OF THE EPITHELINS 1 AND 2

##### 6.4.1. PHYSICAL AND CHEMICAL PROPERTIES

Epithelin 1 and epithelin 2 are resistant to treatment with 1M acetic acid, 1M ammonium hydroxide, 6M urea, 0.01M sodium metaperiodate, to heating at 56°C for 30 minutes, and to treatment with various glycosidases or lipases. However, epithelin activity was sensitive to

reduction, to reduction and 4-vinylpyridine treatment, and to digestion with proteinases such as trypsin, endoproteinase Lys-C, and endoproteinase Glu-C (V8). These results suggest that these factors are proteins containing cysteines in disulfide linkage(s) that are essential for biological activity. These proteins do not contain oligosaccharides and/or lipid moieties that are obligatory for biological activities.

#### 6.4.2. PURIFICATION OF EPITHELIN 1 AND EPITHELIN 2 AND CERTAIN PHYSICAL PROPERTIES

Summaries of the purification of epithelin 1 and epithelin 2 are presented in Table I and Table II, respectively. Both factors were purified to apparent homogeneity by a similar six-step protocol. The early step fractions contain a multiple of growth inhibitory activities on A431 cells. Epithelins 1 and 2 constitute only a very minor fraction of total GIA in early fractions, making it very difficult to quantitate their specific activities at early stages of purification. The specific activity of purified epithelin 1 was about  $2.1 \times 10^4$  units/mg protein, whereas purified epithelin 2 had a much lower specific activity of  $3.8 \times 10^3$  units/mg protein.

**TABLE II**  
Summary of Purification of Epithelin 1 (GIA)

5	Fraction	Protein	GIA	Specific Activity	Yield
		$\mu$ g	Units <sup>1</sup>	Units/mg	%
	Crude	4,550,000	1,283,100 <sup>2</sup>	282	-
	Prep TSK-250	473,000	112,200 <sup>2</sup>	237	-
	Prep. ODS (b)	17,600	14,100	801	100
	Semi Prep. C18				
10	1b	1,510	2,080	1,377	14.8
	2b	3,460	5,460	1,578	38.7
	Anal. Cyano				
	1b	60	713	11,889	3.4
	2b	73	1,190	16,301	8.4
	Anal. Tsk-250				
	1b	41	845	20,609	6.0
15	2b	62	1,305	21,048	9.3

<sup>1</sup> One unit of GIA is the amount of factor required to inhibit <sup>125</sup>I-labeled deoxyuridine incorporation into A431 cells by 50%.

<sup>2</sup> Other growth inhibitory activities are present in these fractions. These values include all activities.

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**TABLE III**  
Summary of Purification of Epithelin 2 (GIA)

5	Fraction	Protein	GIA	Specific Activity	Yield
		$\mu$ g	Units <sup>1</sup>	Units/mg	%
	Crude	4,550,000	1,283,100 <sup>2</sup>	282	-
	Prep TSK-250	473,000	112,200 <sup>2</sup>	237	-
	Prep. ODS (b)	24,500	9,567	432	100
	Semi Prep. C18	4,760	1,190	250	12.4
10	Anal. Cyano	169	460	2,741	4.8
	Anal TSK-250	37	141	3,810	1.5

<sup>1</sup> One unit<sub>125</sub> of GIA is the amount of factor required to inhibit <sup>125</sup>I-labeled deoxyuridine incorporation into A431 cells by 50%.

<sup>2</sup> Other growth inhibitory activities are present in these fractions. These values include all activities.

The molecular weight of epithelin 1 and epithelin 2, as determined by gel permeation chromatography on TSK-250 columns, was ~4,500 and ~3,700, respectively (FIGs. 6, 7 and 10). SPE-epithelin 1 or 2 exhibited a molecular weight of ~13,000 by similar gel permeation chromatography.

FIG. 11 shows an analysis of epithelin 1 and epithelin 2 in an 18% polyacrylamide gel under reducing conditions. Epithelin 1 and epithelin 2 migrated in the gel as single bands with median relative molecular weights of about 5,500 and 6,000, respectively. Similar results were obtained when proteins were electrophoresed under nonreducing conditions. Thus, epithelins are single chain, low molecular weight proteins.

#### 6.4.3. CHEMICAL STRUCTURE OF EPITHELIN 1 AND EPITHELIN 2

The amino acid sequences of epithelin 1 and epithelin 2 were deduced from microsequence analysis of S-pyridylethylated proteins, and fragments generated by endoproteinase Lys-C, Staphylococcal aureus V8 and TPCK trypsin. The amino acid sequences of epithelin 1 and epithelin 2 are presented in FIG. 12.

The protein sequences of epithelin 1 and epithelin 2 were compared with all proteins in the National Biomedical Research Foundation data base (release 22), Genetic Sequence Data Bank (Bolt Beranek and Newman, Los Alamos National Laboratory; release 61) and the European Molecular Biology Laboratory data base (release 20). These computer aided searches revealed that both proteins are novel and do not share any significant homology to any protein in the three data bases.

Epithelin 1 and epithelin 2 are single chain polypeptides of 56 and 57 amino acid residues, respectively, having calculated molecular weights of 6060 and 6094, respectively. Epithelin 1 and epithelin 2 share 47% homology at the amino acid level. Both proteins contain 12 cysteine residues with four double cysteine residues. Thus about 21% of amino acid residues in both proteins are cysteines. Moreover, the spacing of single cysteine residues and double cysteine residues in epithelin 1 and epithelin 2 is identical. Although the number or position of intrachain disulfide bonds in these proteins is not presently known, a similar or identical pattern is expected. Both proteins also contain about 13% hydroxy amino acids (serine and threonine together). The amino acid alignment of epithelin 1 and epithelin 2 is shown in FIG. 12. In addition the conservation of cysteine residues, 15 other residues are conserved between epithelin 1 and epithelin 2.

The hydropathy profiles of epithelins 1 and 2 are presented in FIG. 13. The hydropathy profile of epithelin 1 exhibits some similarity to that of epithelin 2, although epithelin 2 appears to be more hydrophilic than epithelin 1.

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#### 6.4.4. BIOLOGICAL PROPERTIES OF EPITHELIN 1 AND EPITHELIN 2

The inhibition of  $^{125}\text{I}$ -deoxyuridine incorporation into DNA of human epidermoid carcinoma A431 cells by different concentrations of purified epithelin 1 and epithelin 2 is given in FIG. 14. A 50% inhibition of DNA synthesis was seen at 12.8 ng/well of epithelin 1 and 450ng/well of epithelin 2. Thus, a 50% inhibition occurred at approximately 21 nM concentration of epithelin 1 and approximately 0.75  $\mu\text{M}$  concentration of epithelin 2. Epithelin 1 is therefore about 36 times more potent than epithelin 2 in this assay.

The effect of epithelin on the incorporation of  $^{125}\text{I}$ -deoxyuridine into DNA of various tumor and non-tumor human cell lines, as well as several non-human cell lines, was investigated. Epithelin 1 (20 ng/ml, maximum dose tested) slightly inhibited the growth of human colon carcinoma cell line HCT 116, while epithelin 2 (270 ng/ml, maximum dose tested) did not show any effect on this cell line. Both proteins significantly inhibited the  $^{125}\text{I}$ -deoxyuridine incorporation into DNA of mink lung CCL 64 cells and monkey kidney COS1 cells. Neither protein exhibited any significant effect on human fibroblasts and several other human tumor cells at the maximum dose tested (20 ng/ml for epithelin 1 and 270 ng/ml for epithelin 2).

The effects of various concentrations of epithelin 1 and epithelin 2 on the incorporation of  $^{125}\text{I}$ -deoxyuridine into DNA of murine keratinocytes (Balb/MK cells) was investigated. Data are presented in FIG. 15A.

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Epithelin 1 stimulated the  $^{125}\text{I}$ -deoxyuridine incorporation in a dose-dependent manner, while epithelin 2 did not show any significant effect. However, epithelin 2 inhibited epithelin 1 elicited incorporation of  $^{125}\text{I}$ -deoxyuridine incorporation to Balb/MK cells (FIG. 15B). In this regard, a 50% inhibition was observed at ~21 nM epithelin 2. Thus, epithelin 2 antagonizes the effect of epithelin 1 in this system.

The continued growth of a murine keratinocyte cell line, Balb/MK, is dependent on EGF, TGF $\alpha$  or amphiregulin (AR). Balb/MK cells did not proliferate in the absence of EGF or epithelin 1 (FIG. 16A), whereas epithelin 2 did not exhibit any significant effect on the growth of these cells. However, epithelin 2 inhibited the epithelin 1-induced growth of Balb/MK cells in a dose-dependent manner (FIG. 16B); a 50% inhibition was observed at ~7 nM epithelin 2. EGF or TGF $\alpha$  induces anchorage-independent growth of rat kidney cells NRK-SA6 in the presence of TGF $\beta$  (Roberts et al., 1981, Proc. Natl. Acad. Sci. USA 78:5339-5344). Like EGF, epithelin 1 induced the anchorage-independent growth of NRK cells in a dose-dependent manner (FIG. 17), while epithelin 2 did not. Epithelin 2 (at a concentration of ~85 nM) inhibited about 50% of the epithelin 1-induced colony formation in soft agar. Furthermore, epithelin 1, but not epithelin 2, exhibited mitogenic activity on NRK cells in monolayer.

Neither epithelin 1 nor epithelin 2 significantly affected the binding of  $^{125}\text{I}$ -EGF to its receptors, suggesting that the epithelins do not mediate their biological effects through EGF receptors.



7. EXAMPLE: cDNA CLONING OF THE  
EPITHELIN PRECURSOR AND TRANSIENT  
EXPRESSION OF PRECURSOR AND MATURE FORMS

7.1. PCR cDNA CLONING

5 Two pools of degenerate oligonucleotides were  
synthesized based on the peptide sequences KTQCPDD and  
HCCPQDT from epithelin 2 (the pools contained 256 and 128  
degenerate oligonucleotides in the sense and antisense  
10 orientation, respectively). These oligonucleotides were  
used as primers in a 40 cycle PCR amplification with a  
single stranded cDNA template derived from rat kidney RNA  
primed with XSCT17, an oligonucleotide containing a T<sub>17</sub>  
track on its 3'-end (Plowman et al, 1990, Proc. Natl. Acad.  
15 Sci. U.S.A. 87:4905-08). An oligonucleotide probe, based  
on the epithelin 2 sequence KYGCCPMP (256 fold degenerate  
23-mer), was labeled with [ $\gamma$ -<sup>32</sup>P]ATP and used to screen the  
PCR products.

Hybridization of this probe to a Southern blot of  
20 the PCR products revealed two major bands of 120 base pairs  
(bp) and 600 bp, and a minor band of 325 bp. These  
fragments were subcloned and sequenced, revealing that they  
all had a common 5' end encoding epithelin 2. The 325 bp  
fragment had an open reading frame that encoded both  
25 epithelin 1 and 2, whereas the 600 bp band extended further  
3' and contained an additional copy of the cysteine-rich  
motif. Therefore, epithelin 1 and 2 appear to be tandemly  
arranged products of a single transcript. These same PCR  
primers were used to isolate similar fragments of the  
30 epithelin precursor from human, bovine, mouse, and chicken  
cDNA, demonstrating strong evolutionary conservation of the  
cysteine-rich motif.

The complete epithelin cDNAs were obtained from rat, mouse, and human sources by using a PCR protocol to isolate the 5' and 3' ends of messages that have a known central sequence, and by screening  $\lambda$ gt10 libraries. In particular, a PCR strategy with exact epithelin primers oriented in the 3' and 5' directions in combination with primers that anneal to the natural poly(A) tail, or a synthetic poly(A) track added onto the 5' extended cDNA was employed (Plowman et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:4905-08). A rat kidney cDNA library was constructed in  $\lambda$ gt10 (Plowman et al., 1990, Mol. Cell Biol. 10:1969-81), and a full length rat epithelin cDNA was isolated by screening  $2.0 \times 10^5$  recombinants with PCR generated epithelin probes. These probes were also used to obtain the mouse epithelin gene from a mouse T-cell genomic library (Stratagene, La Jolla, CA). Several PCR-generated clones, rat cDNA clones, and the mouse genomic clones were sequenced on both strands by using T7 polymerase with oligonucleotide primers (Tabor and Richardson 1987, Proc. Natl. Acad. Sci. U.S.A. 84:4767-71).

The composite sequence of the rat epithelin cDNA (FIG. 18) is 2150 bp long, which closely approximates the 2.3 kilobase transcript seen by northern analysis with rat kidney mRNA. The sequence predicts a 589-residue preprotein with a 30 bp 5'-untranslated region and a 343 bp 3'-untranslated region. The first AUG is followed by an N-terminal hydrophobic signal peptide of 16 amino acids and the 573 amino acid mature epithelin precursor with a predicted  $M_r$  of 61,597. The precursor has no transmembrane domain, 3 potential N-linked glycosylation sites, and 88 cysteine residues. The epithelin precursor has a highly repetitive organization (FIG. 19) containing 7 tandem copies of a 55-57 amino acid consensus motif:  $VXCX_5-CX_5CCX_8CCX_6CCXDX_2HCCPX_4CX_5-6CX_2$ . Two of the repeats represent the known active molecules, epithelin 1 and 2.

The predicted amino acid sequences of the mouse and human epithelin precursors contain 589 and 593 residues, respectively, and exhibit 86% (mouse) and 75% (human) sequence identity with the rat protein (FIG 21A).

5 Additional isoforms of epithelin may also exist, since one rat cDNA clone has a deletion of 234bp (FIG 21A, amino acids 398-475), maintained the reading frame, and generated a chimeric motif. This deletion is likely the result of alternative splicing since its boundaries map precisely to the location of exon-intron junctions in the mouse  
10 epithelin gene.

Comparison of the epithelin sequence with available protein and DNA sequence databases reveals three regions with homology to known proteins. The first 41  
15 amino acids following the signal sequence of the epithelin precursor contains 6 cysteine residues arranged in a pattern similar to the N-terminal half of the other seven cystein-rich motifs. The sequence CPDGQFCPVACC is completely conserved between human, rat and mouse epithelins, and conforms to a consensus pattern present in  
20 a family of snake toxins (Dufton, 1984, J. Mol. Evol. 20:128-34). A second region of homology exists within three of the cystein-rich motifs of rat epithelin (CCX<sub>2</sub>HX<sub>2</sub>C), and conforms to a consensus surrounding an active site of phospholipase A2 (Gomez et al., 1989, J.  
25 Eur. J. Biochem. 186:23-33). Finally, an extended homology exists between the 12 cysteine motif of epithelin and the C-terminal regulatory domain of a tomato thiol protease (Fig. 21D). This noncatalytic domain has been hypothesized to regulate the protease activity by binding to heavy  
30 metals. The alternating cysteine and histidine residues in the epithelin precursor is reminiscent of metal-binding domains of a variety of proteins, although the epithelin motif does not conform to that of any known metal-binding  
35 consensus. Northern analysis demonstrates that the 2.3

kilobase epithelin transcript is ubiquitously expressed, and is predominant in the adult kidney, placenta, heart, duodenum, colon, and cerebral cortex. In addition, it is present in a wide variety of epithelial tumor cell lines. Southern analysis indicates that the epithelin precursor is encoded by a single copy gene. These results suggest a post-transcriptional mechanism for the generation of active molecules from the epithelin precursor.

## 7.2. EXPRESSION IN COS CELLS

The biochemical properties of rat epithelin was determined by inserting its complete coding sequence into an expression vector under the control of the cytomegalovirus immediate-early promoter (Seed and Aruffo, 1987, Proc. Natl. Acad. Sci. U.S.A. 84:3365-69). Specifically, a 1.6 kb fragment containing the complete rat epithelin coding sequence was inserted into a cDM8 based expression vector, generating the expression vector crEPN1.6. A similar construct, crEPN1.4, was prepared by insertion of a 1.35 kb fragment from an epithelin cDNA that has a single exon deletion, thereby eliminating amino acids 398-475. The secretion plasmids, c $\beta$ rEPN1 and c $\beta$ rEPN2, were constructed by ligating a synthetic simian TGF- $\beta$ 1 signal sequence contained on the oligonucleotides

```

                    M P P S G L R L L P L L
25  5' AGCTTCTGCAGGGGCGGGGCTCCCC ATGCCGCCCTCCGGGCTGCGGCTGCTGCCGCTGCTG
    3'  AGACGTCCCCGCCCGGAGGGGG TACGGCGGGAGGCCCGACGCCGACGACGGCGACGAC

    L P L L W L L V L T P S R P A A
    CTACCGCTGCTGTGGCTACTGGTGCTGACGCCTAGCCGGCCGGCCGC 3'
    GATGGCGACGACACCGATGACCACGACTGCGGATCGGCCGGCCGG 5'

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and PCR generated SacII-XbaI fragments containing the coding sequence of epithelin 1 and 2 into the cDM8SII plasmid digested with HindIII and XbaI. cDM8SII is a modified cDM8 vector, where the unique SacII site has been

eliminated with Klenow. The new SacII site places a final glycine of the signal peptide in frame with the epithelin coding sequence. The expression plasmids were grown in competent MC1061/P3 bacteria, and introduced into COS-1 cells using the DEAE-dextran method (Seed and Aruffo, 1987, Proc. Natl. Acad. Sci. U.S.A. 84:3365-69). Forty-eight hours after transfection, the cells were washed with phosphate buffered saline, and labeled for 16 hours in serum free MEM (4 ml per 100 mm plate) supplemented with 250 $\mu$ Ci/ml <sup>35</sup>S-cysteine (1100 Ci/mmol, New England Nuclear). Labeled supernatants were dialyzed against 0.1 N acetic acid, dried, and 1 ml equivalents run on 10% or 15% SDS-polyacrylamide gels.

The transiently expressed protein was readily detected by SDS-PAGE analysis of <sup>35</sup>S-cysteine labeled supernatants. The recombinant protein had a molecular mass of about 75K, slightly more than the predicted 62K, possibly due to glycosylation (FIG 26A, lane 1). A similar construct encoding an epithelin isoform, but lacking an exon in the C-terminal region, migrated with average  $M_r$  of 68K, commensurate with the deletion of 78 amino acids. There was no evidence of the precursor being processed into smaller forms. To express the mature recombinant epithelin 1 and 2 proteins, their coding regions were placed into an expression vector behind a synthetic signal peptide sequence (c $\beta$ rEPN1 and c $\beta$ rEPN2, respectively), which resulted in secretion of the 6K proteins from COS cells (FIG. 26B). The cells transfected with these constructs were growth inhibited and exhibited an altered morphology with many cells showing a signet ring appearance, compared with the intact monolayer seen on a mock transfection. Supernatant from the c $\beta$ rEPN1 transfection was partially purified on a Bio-Sil TSK-250 column (Shoyab et al., 1990, 87:4905-09) and fractions were assayed for growth inhibitory activity (GIA) on A431 cells. These cells

secreted active epithelin 1 (approximately 25 GIA units/100 mm plate) whereas the supernatant from mock transfected cells had no detectable activity (one GIA unit corresponds to the amount of material required for 50% inhibition of cell growth).

5           Epithelin 1 has an inhibitory effect on A431 cells and it acts as mitogen on several normal epithelial cell lines (See Section 6., supra). In addition, epithelin 1 can induce anchorage-independent growth of rat kidney  
10 fibroblasts in the presence of transforming growth factor  $\beta$  (FIG. 17). In contrast, epithelin 2 has no effect on the growth of rodent keratinocytes of fibroblasts and it opposed the mitogenic effects of epithelin 1 (in a dose dependent manner) in both of these systems (FIG. 17). The  
15 unprocessed epithelin precursor had no activity in any of these assays. Perhaps the intact precursor serves an entirely different role than the processed forms, such as chelating metal ions, or regulation of proteases and phospholipases.

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## WHAT IS CLAIMED IS:

1. A human epithelin precursor comprising the amino acid sequence substantially as depicted in FIG. 22 from about amino acid residue numbers 1 to 593.
2. Human epithelin 1 comprising the amino acid sequence substantially as depicted in FIG. 22 from about amino acid residue numbers 282 to 337.
3. Human epithelin 2 comprising the amino acid sequence substantially as depicted in FIG. 22 from about amino acid residue numbers 206 to 262.
4. A human epithelin comprising the amino acid sequence substantially as depicted in FIG. 22 from about amino acid residue numbers 59 to 114.
5. A human epithelin comprising the amino acid sequence substantially as depicted in FIG. 22 from about amino acid residue numbers 124 to 180.
6. A human epithelin comprising the amino acid sequence substantially as depicted in FIG. 22 from about amino acid residue numbers 364 to 418.
7. A human epithelin comprising the amino acid sequence substantially as depicted in FIG. 22 from about amino acid residue numbers 442 to 497.
8. A human epithelin comprising the amino acid sequence substantially as depicted in FIG. 22 from about amino acid residue numbers 519 to 574.

9. A nucleic acid molecule encoding a human epithelin precursor comprising the nucleotide sequence substantially as depicted in FIG. 22 from about nucleotide residue numbers 41 to 1819.

5 10. An anti-sense ribonucleic acid molecule complementary to the nucleic acid molecule of claim 9.

10 11. A rat epithelin precursor comprising the amino acid sequence substantially as depicted in FIG. 18 from about amino acid residue numbers 1 to 589.

15 12. Rat epithelin 1 comprising the amino acid sequence substantially as depicted in FIG. 18 from about amino acid residue numbers 280 to 335.

13. Rat epithelin 2 comprising the amino acid sequence substantially as depicted in FIG. 18 from about amino acid residue numbers 205 to 261.

20 14. A rat epithelin comprising the amino acid sequence substantially as depicted in FIG. 18 from about amino acid residue numbers 59 to 114.

25 15. A rat epithelin comprising the amino acid sequence substantially as depicted in FIG. 18 from about amino acid residue numbers 123 to 179.

30 16. A rat epithelin comprising the amino acid sequence substantially as depicted in FIG. 18 from about amino acid residue numbers 362 to 416.

35 17. A rat epithelin comprising the amino acid sequence substantially as depicted in FIG. 18 from amino acid residue numbers 440 to 495.



18. A rat epithelin comprising the amino acid sequence substantially as depicted in FIG. 18 from amino acid residue numbers 515 to 570.

5 19. A nucleic acid molecule encoding a rat epithelin precursor comprising the nucleotide sequence substantially as depicted in FIG. 18 from about about nucleotide residue numbers 31 to 1797.

10 20. An anti-sense ribonucleic acid molecule complementary to the nucleic acid molecule according to claim 19.

15 21. A mouse epithelin precursor comprising the amino acid sequence substantially as depicted in FIG. 23 from about amino acid residue numbers 1 to 589.

20 22. Mouse epithelin 1 comprising the amino acid sequence substantially as depicted in FIG. 23 from about amino acid residue numbers 280 to 335.

23. Mouse epithelin 2 comprising the amino acid sequence substantially as depicted in FIG. 23 from about amino acid residue numbers 205 to 261.

25 24. A mouse epithelin comprising the amino acid sequence substantially as depicted in FIG. 23 from about amino acid residue numbers 59 to 114.

30 25. A mouse epithelin comprising the amino acid sequence substantially as depicted in FIG. 23 from about amino acid residue numbers 123 to 179.

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26. A mouse epithelin comprising the amino acid sequence substantially as depicted in FIG. 23 from about amino acid residue numbers 362 to 416.

5 27. A mouse epithelin comprising the amino acid sequence substantially as depicted in FIG. 23 from amino acid residue numbers 440 to 495.

10 28. A mouse epithelin comprising the amino acid sequence substantially as depicted in FIG. 23 from amino acid residue numbers 515 to 570.

15 29. A nucleic acid molecule encoding a mouse epithelin precursor comprising the nucleotide sequence substantially as depicted in FIG. 23 from about nucleotide residue numbers 8 to 1774.

20 30. An anti-sense ribonucleic acid molecule complementary to the nucleic acid molecule according to claim 29.

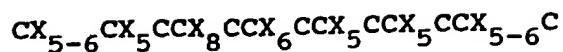
31. A polypeptide comprising the amino acid sequence:

25  $X_2CX_{5-6}CX_5CCX_8CCX_6CCXDX_2HCCPX_4CX_{5-6}CX_2$

wherein C is Cysteine, D is Aspartic Acid, H is Histidine, P is Proline, and X is any amino acid.

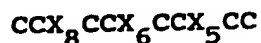
30 32. A polypeptide containing an amino acid sequence comprising:

35



wherein C is Cysteine and X is any amino acid.

- 5           33. A polypeptide containing an amino acid  
sequence comprising:



10           wherein C is Cysteine and X is any amino acid.

34. A method of inhibiting the growth of  
neoplastic cells comprising contacting the cells with at  
least one epithelin effective at inhibiting neoplastic cell  
15           growth.

          35. The method according to claim 34 wherein the  
epithelin is epithelin 1.

20           36. The method according to claim 34 wherein the  
epithelin is epithelin 2.

          37. A method of treating neoplasia in an animal  
comprising administering to the animal at least one  
epithelin in an amount effective at inhibiting neoplastic  
25           cell proliferation.

          38. The method according to claim 37 wherein the  
epithelin is epithelin 1.

30           39. The method according to claim 37 wherein the  
epithelin is epithelin 2.

40. A method of promoting wound healing comprising administering to the locus of a wound at least one epithelin effective at stimulating cell growth.

5 41. The method according to claim 40 wherein the epithelin is epithelin 1.

42. A method of promoting wound healing comprising administering to the locus of a wound an epithelin 2 antagonist at a dose effective at inhibiting  
10 the epithelin 1-antagonizing activity of epithelin 2.

43. The method according to claim 42 wherein the epithelin 2 antagonist is coadministered with an epithelin capable of promoting cell growth.  
15

44. The method according to claim 42 wherein the epithelin 2 antagonist is coadministered with epithelin 1.

20 45. The method according to claim 42 wherein the epithelin 2 antagonist comprises an anti-epithelin 2 antibody.

25 46. The method according to claim 45 wherein the anti-epithelin 2 antibody is coadministered with an epithelin capable of promoting cell growth.

30 47. The method according to claim 45 wherein the anti-epithelin 2 antibody is coadministered with epithelin 1.

35 48. A method of treating psoriasis comprising administering to an individual suffering from psoriasis a composition effective at inhibiting epithelin growth stimulatory activity.

49. The method according to claim 48 wherein the composition comprises an epithelin 1 antagonist.

50. The method according to claim 49 wherein the epithelin 1 antagonist comprises epithelin 2.

5

51. The method according to claim 49 wherein the epithelin 1 antagonist comprises an anti-epithelin 1 antibody.

10

15

20

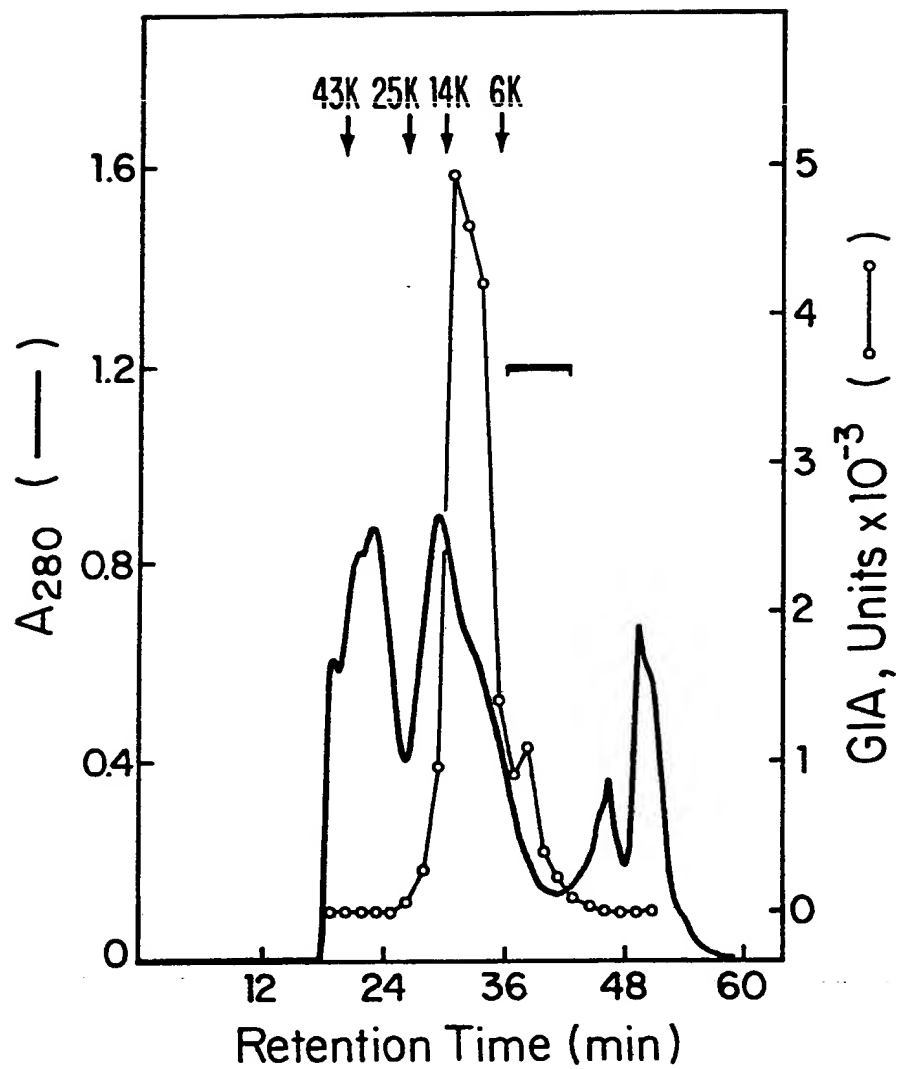
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35

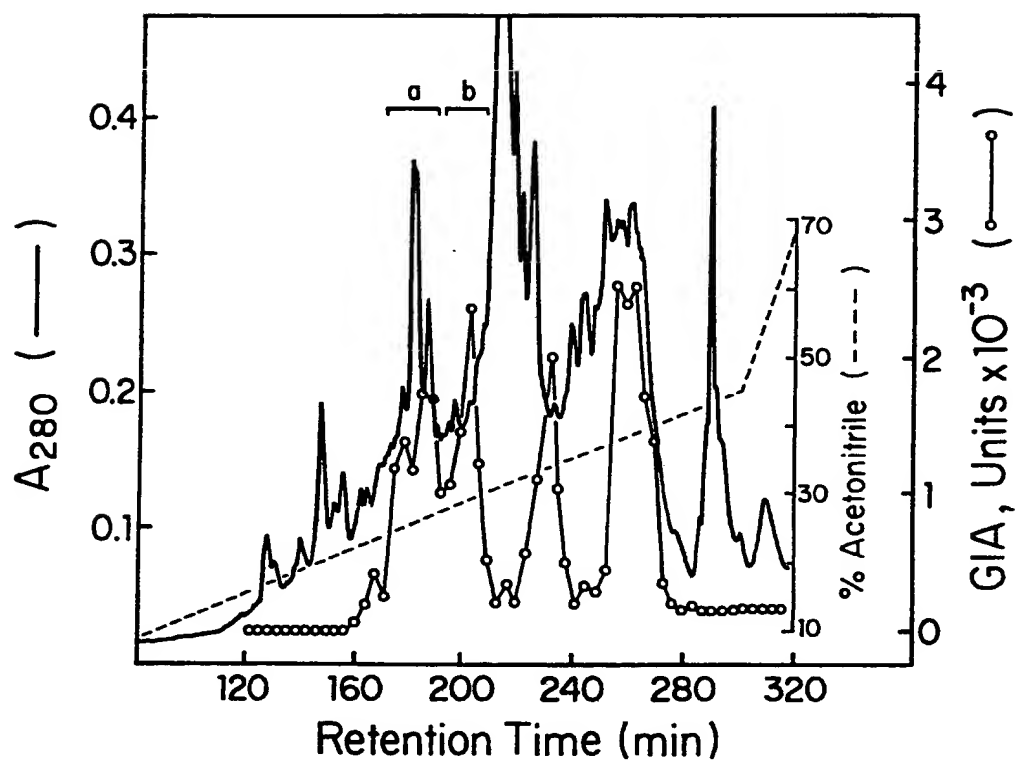
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Figure 1



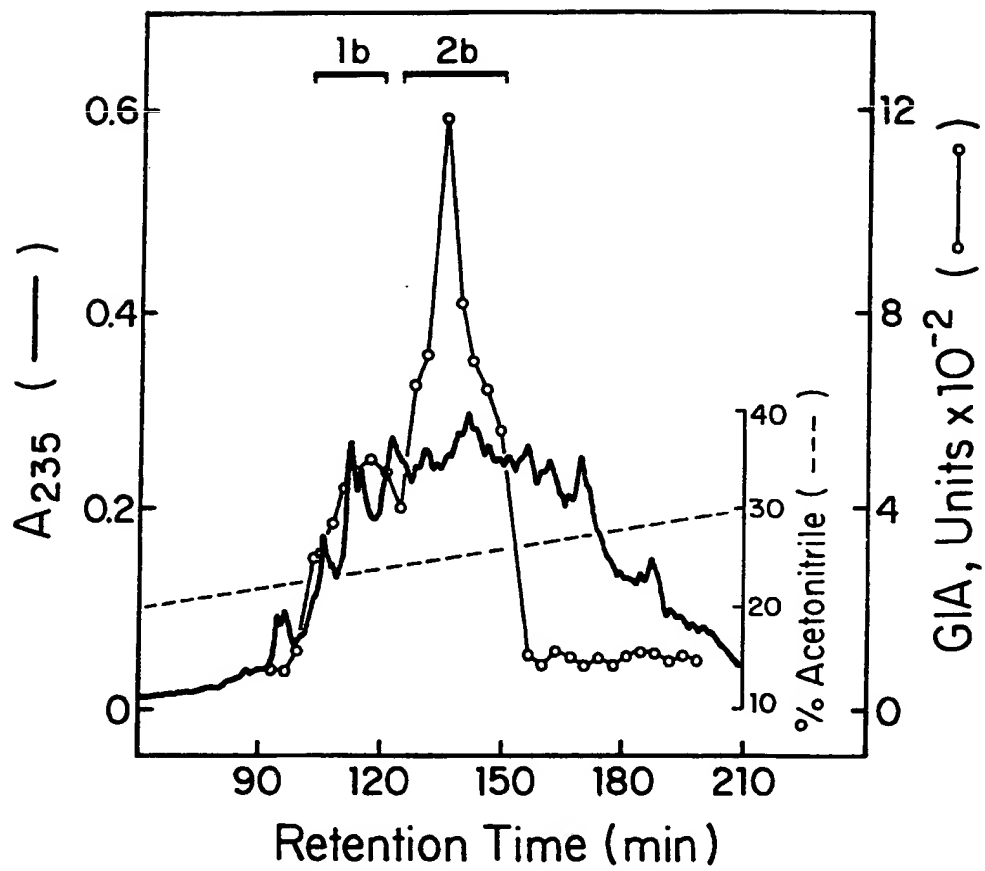
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Figure 2



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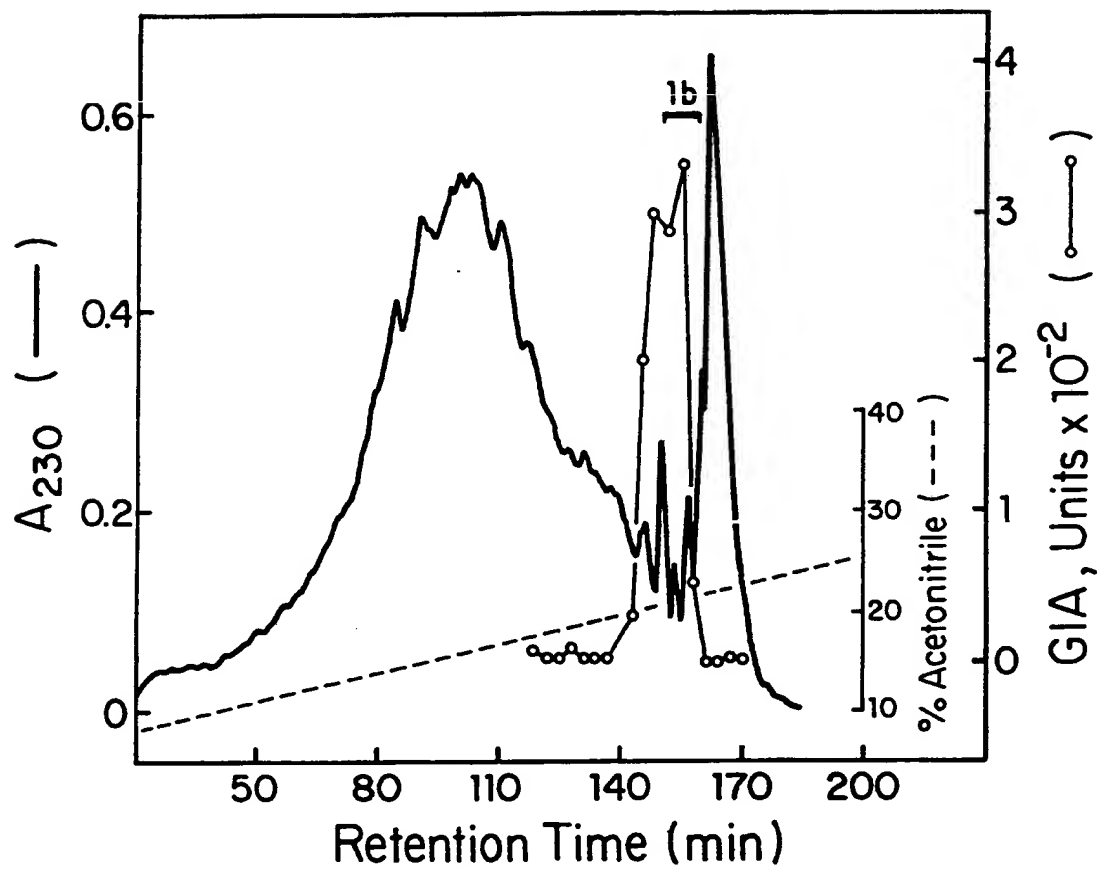
Figure 3





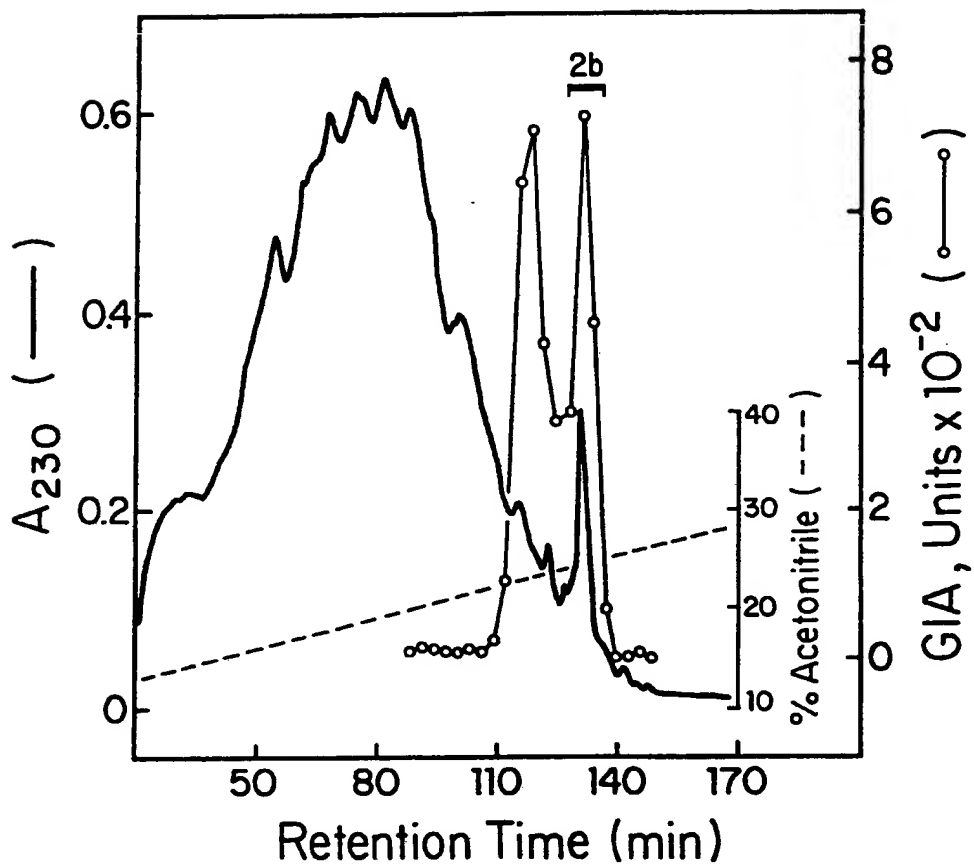
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Figure 4



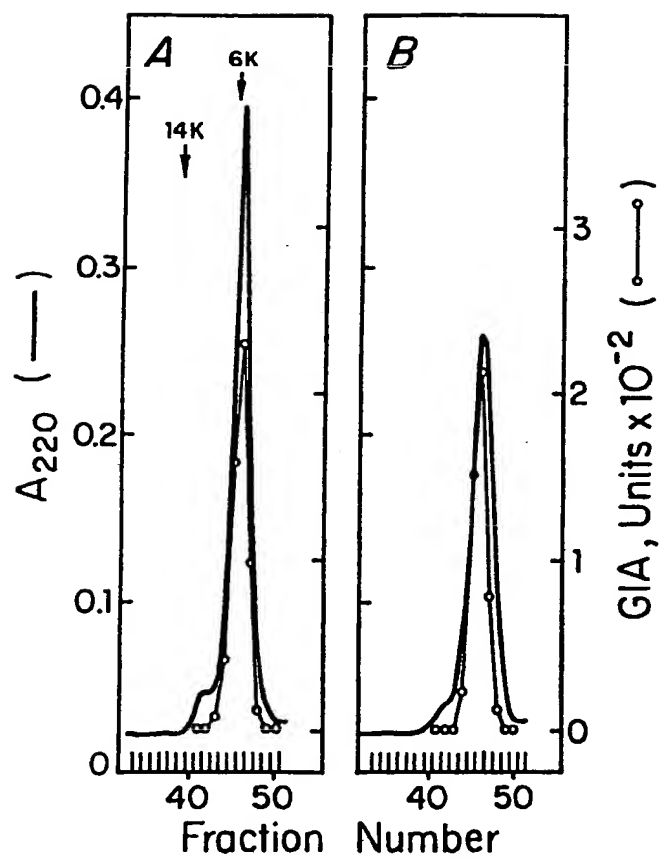
5/36

Figure 5



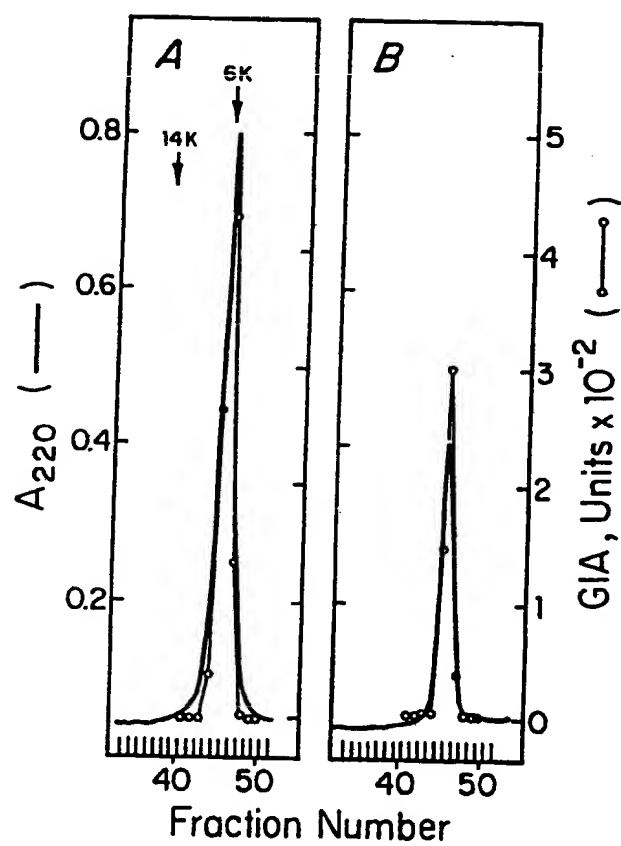
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Figure 6



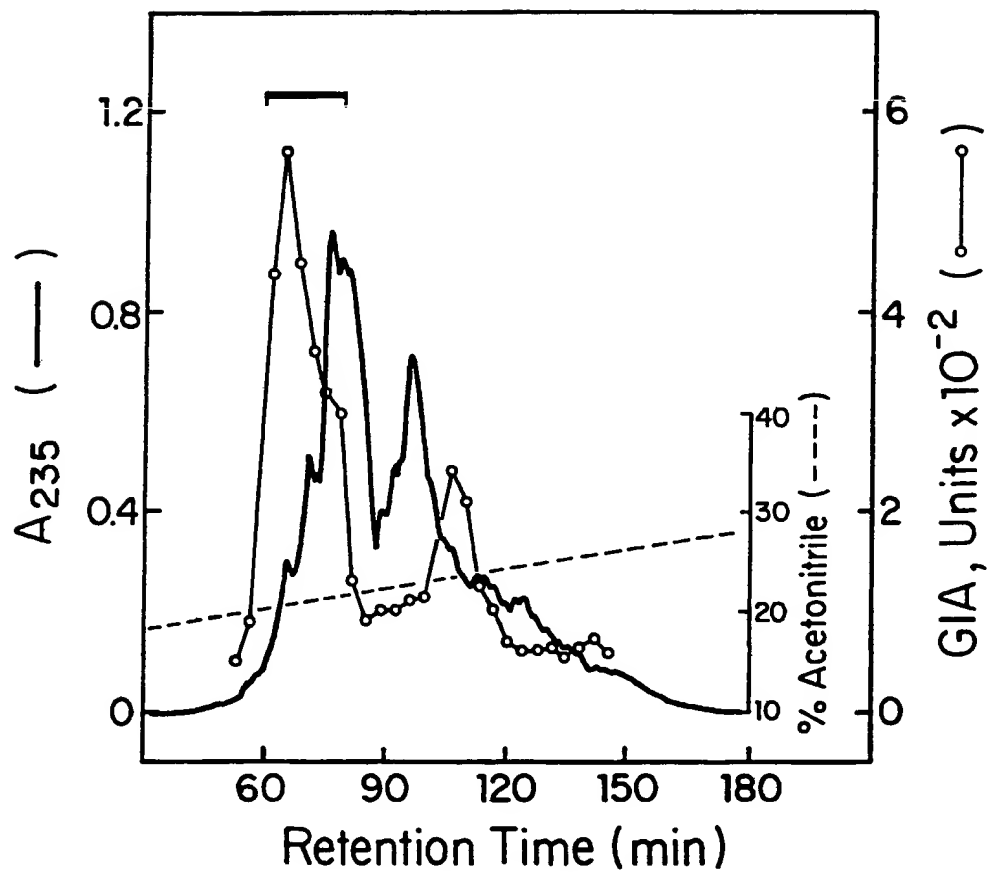
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Figure 7



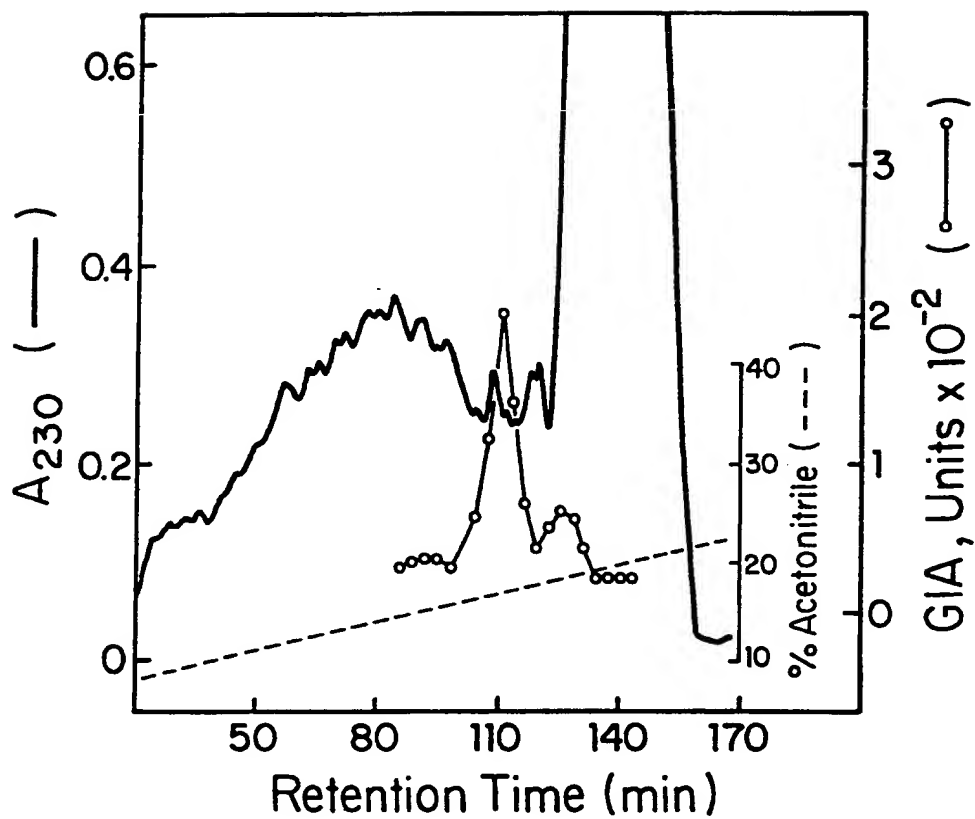
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Figure 8



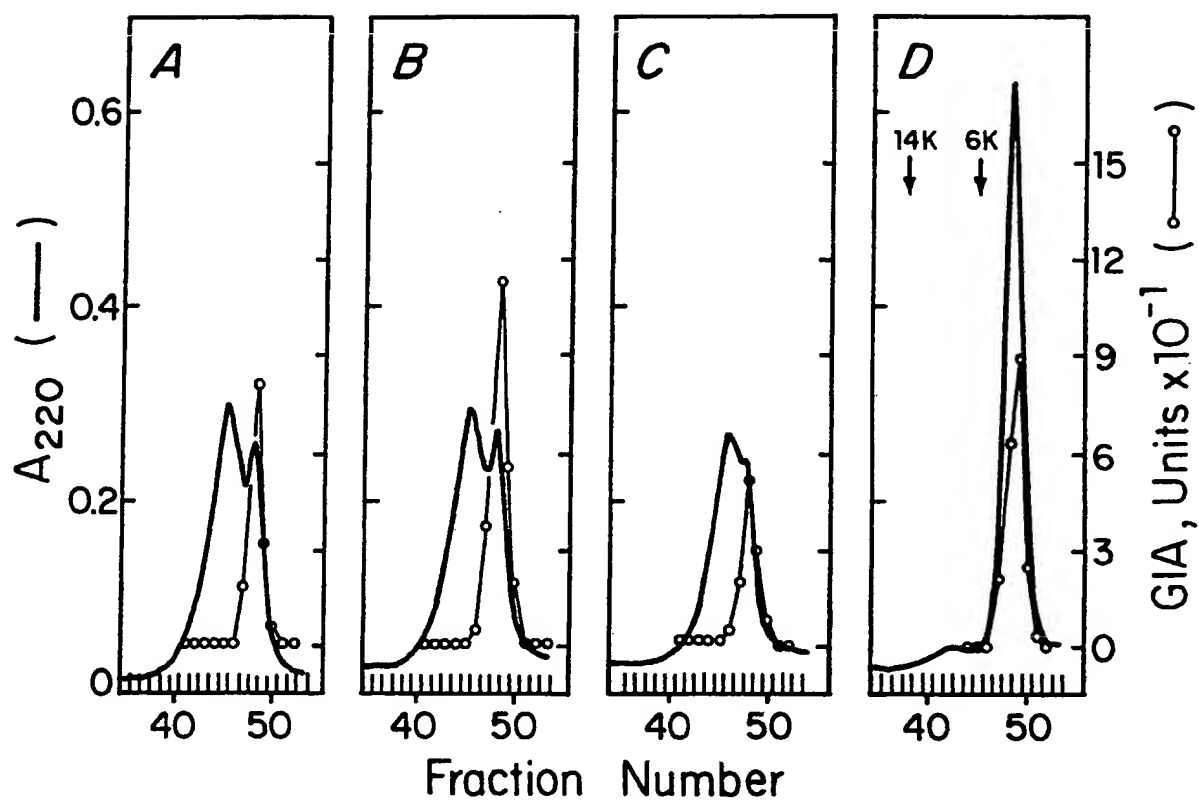
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Figure 9



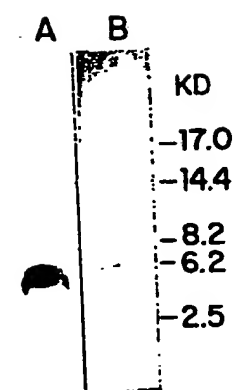
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Figure 10



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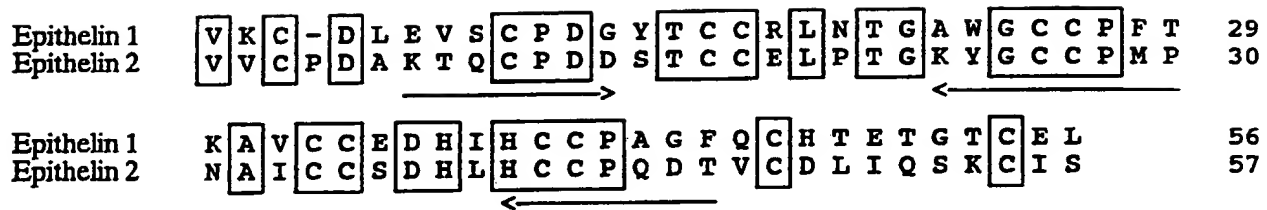
Figure 11





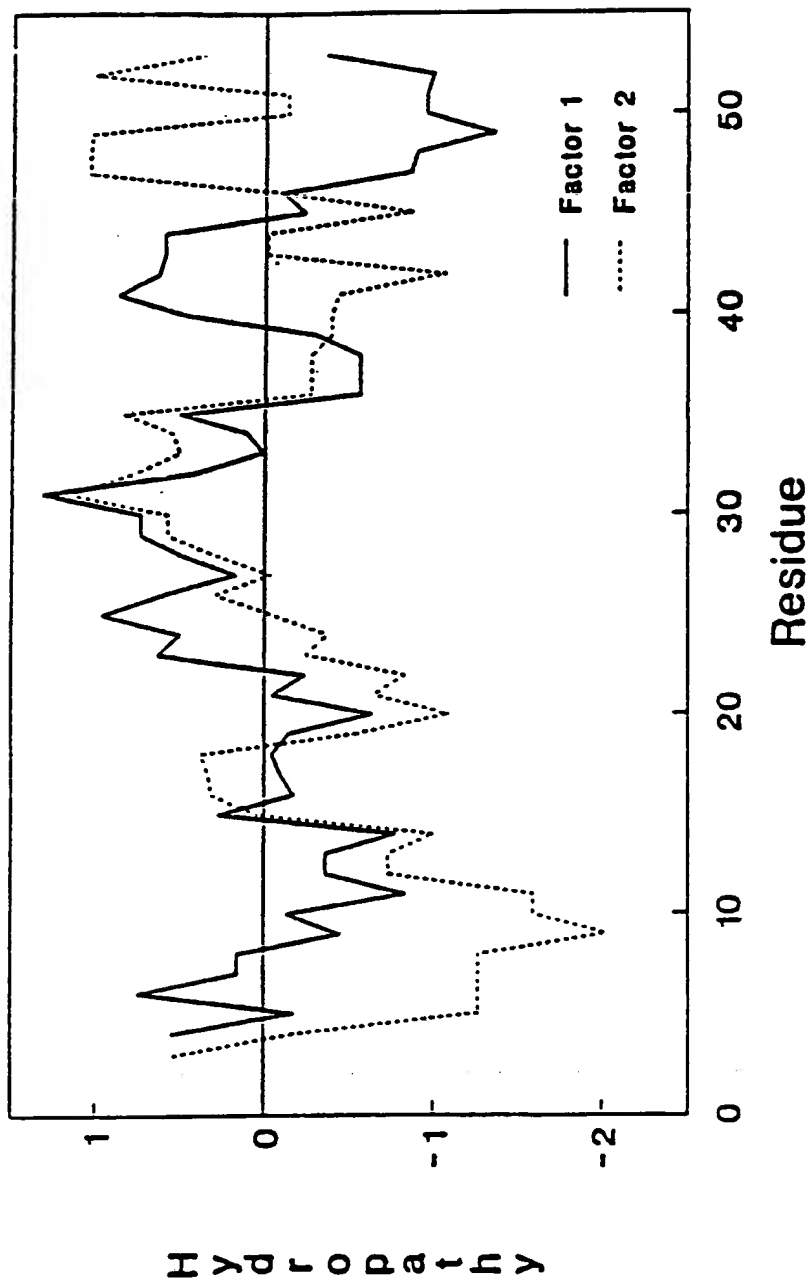
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Figure 12



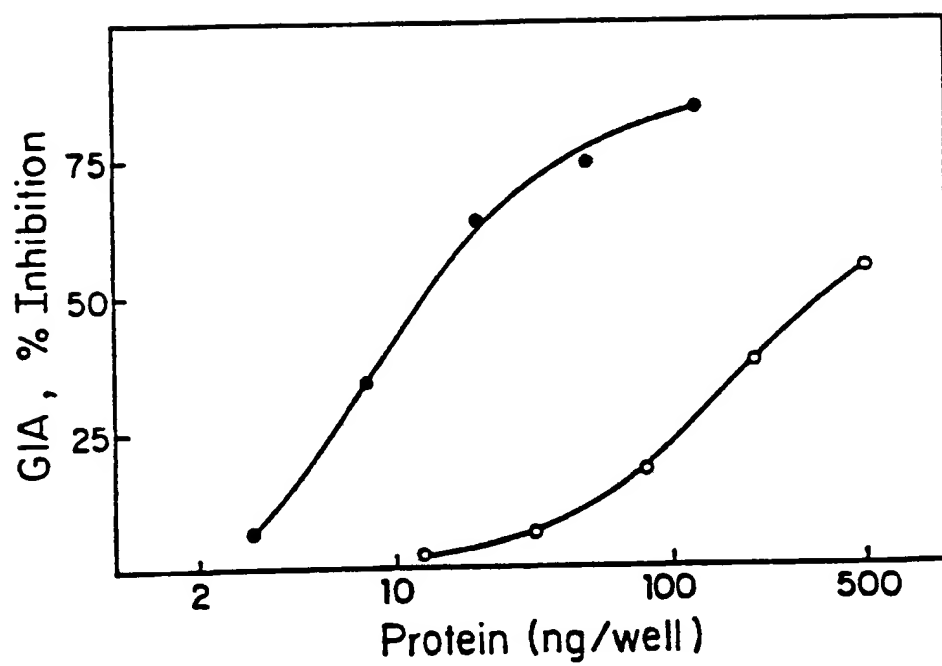
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Figure 13



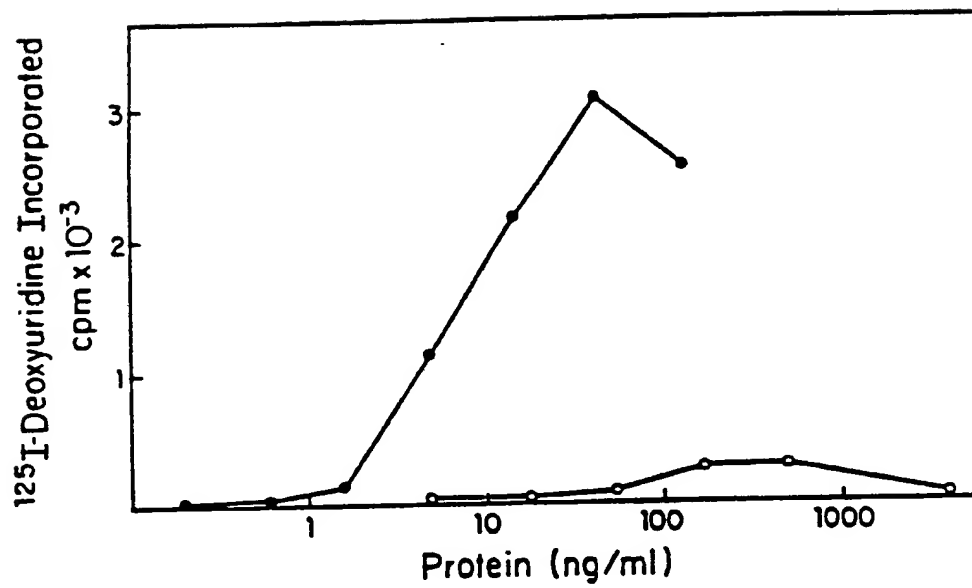
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Figure 14



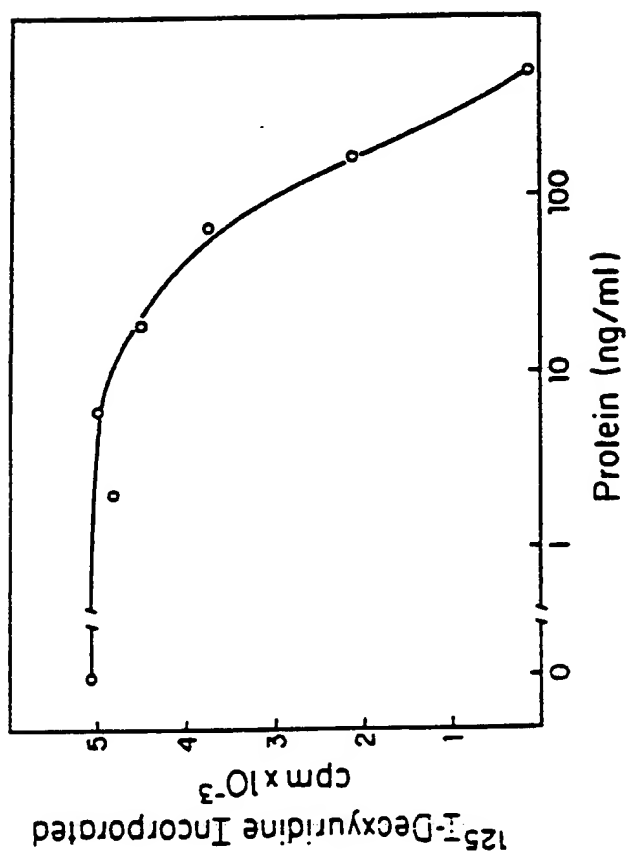
15136

Figure 15A



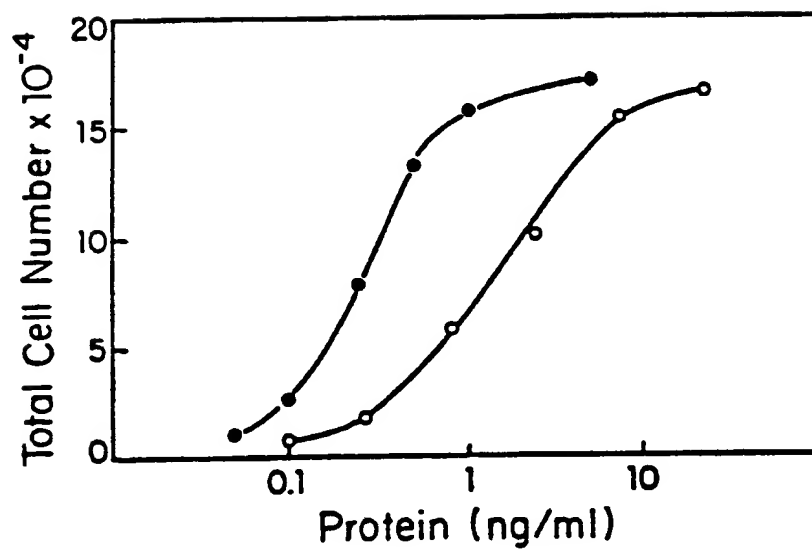
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Figure 15B



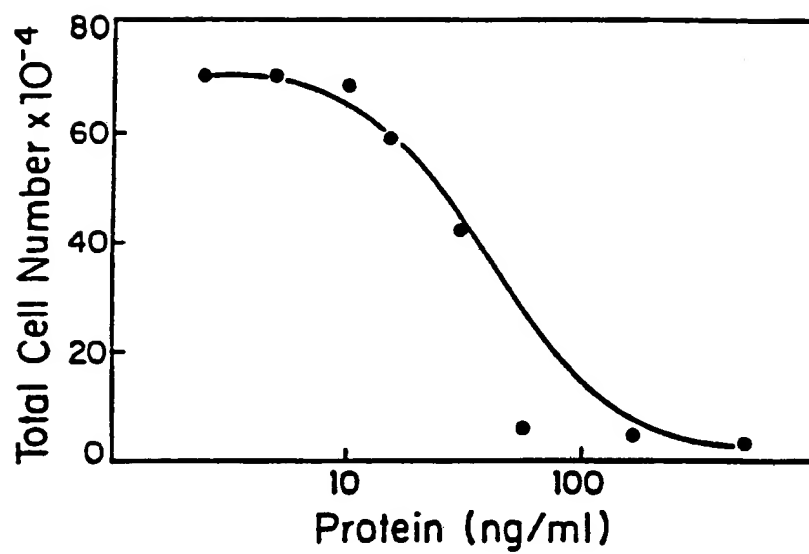
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Figure 16A



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Figure 16B



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Figure 17

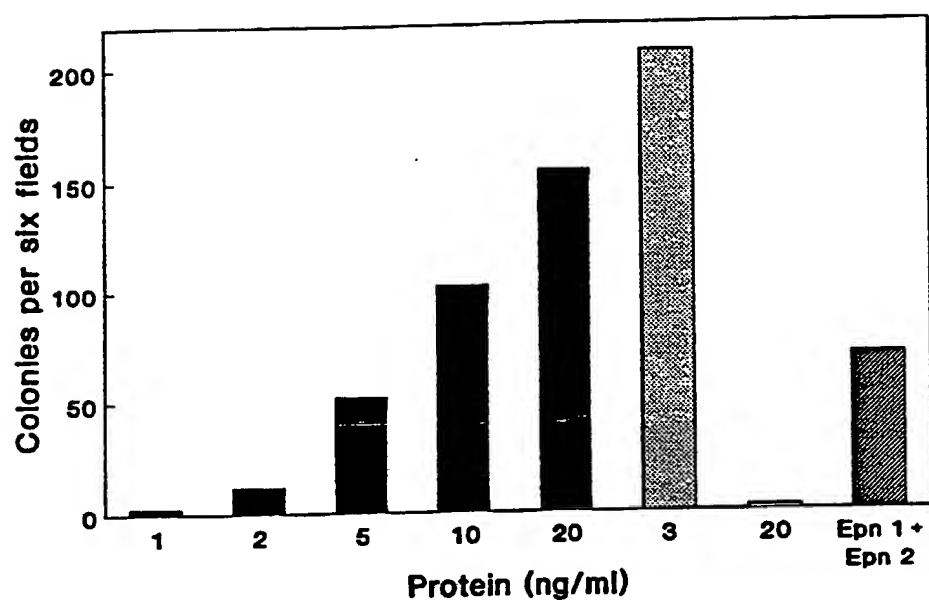




Figure 18

M W I L V S W L A L V A R L V A G T Q C  
ATGTGGATCCTGGTGAGCTGGCTGGCCTTAGTGGCAAGGCTGGTGGCTGGAACACAGTGC 90

P D G Q F C P V A C C L D Q G G A N Y S  
CCAGATGGTCAATTCTGCCCTGTTGCCTGCTGCCTTGACCAGGGAGGAGCCAACTACAGC 150

C C N P L L D T W P I I T S R R L D G S  
TGCTGTAACCCTCTTCTGGACACATGGCCTATAATAACGAGCCGTCGTCTAGATGGCTCC 210

C Q I R D H C P D G Y S C L L T V S G T  
TGCCAGATCCGTGACCACTGTCCTGATGGCTACTCTTGTCTTCTCACTGTGTCTGGGACT 270

S S C C P F S E G V S C D D G Q H C C P  
TCCAGCTGCTGCCCCGTTCTCTGAGGGTGTATCTTGTGATGATGGCCAGCACTGCTGCCCC 330

R G F H C S A D G K S C S Q I S D S L L  
CGGGGCTTCCACTGTAGTGC GGATGGGAAATCCTGCTCTCAGATATCAGATAGCCTCTTG 390

G A V Q C P G S Q F E C P D S A T C C I  
GGTGTGTCCAGTGTCTGGTAGCCAGTTCGAATGTCTGACTCCGCCACCTGCTGTATT 450

M I D G S W G C C P M P Q A S C C E D R  
ATGATTGATGGTTCTG GGGGTGCTGCCCCATGCCCCAGGCCTCTTGCTGTGAAGACAGA 510

V H C C P H G A S C D L V H T R C I S P  
GTGCATTGCTGTCCCCACGGGGCCTCCTGTGACCTGGTTTCACACGCGATGCATTTCACCC 570

T G T H P L L K K F P A Q R T N R A V A  
ACGGGCACCCACCCCTTACTAAAGAAATTCCCCGCACAAAGGACCAACAGGGCAGTGGCT 630

F P F S V V C P D A K T Q C P D D S T C  
TTCCCTTTTCCGTGGTGTGCCCTGATGCTAAGACCCAGTGGCCTGATGACTCTACCTGC 690

C E L P T G K Y G C C P M P N A I C C S  
TGTGAGCTACCCACTGGGAAGTATGGCTGTTGTCCAATGCCCAACGCCATCTGCTGTTCC 750

D H L H C C P Q D T V C D L I Q S K C I  
GACCACCTGCACTGCTGCCCCCAGGACACTGTATGTGACCTGATCCAGAGCAAGTGCATA 810

S K D Y T T D L M T K L P G Y P V N E V  
TCCAAGGACTACACCACAGATCTCATGACCAAGCTGCCTGGATACCCAGTGAATGAGGTG 870

K C D L E V S C P D G Y T C C R L N T G  
AAGTGC GACTTGGAGGTGAGCTGTCCTGATGGCTACACCTGCTGCCGCCTCAACACTGGG 930

A W G C C P F T K A V C C E D H I H C C  
GCCTGGGGCTGCTGTCCATTACCAAGGCTGTGTGTTGTGAAGACCACATTCACTGCTGC 990

P A G F Q C H T E T G T C E L G V L Q V  
CCAGCCGGGTTTCAGTGTACACAGAGACAGGAACCTGTGAACTGGGAGTCCTTCAGGTA 1050

P W M K K V T A S L S L P D P Q I L K N  
CCCTGGATGAAAAAGGTCACGGCCTCCCTCAGCCTGCCAGACCCACAGATCTTGAAGAAT 1110

D V P C D D F S S C P S N N T C C R L S  
GATGTCCCCTGTGATGACTTCAGTAGCTGTCCTTCTAACAATACCTGCTGCAGACTCAGT 1170

S G D W G C C P I P E A V C C L D H Q H  
TCTGGGGACTGGGGCTGCTGTCCCATCCCAGAGGCTGTCTGCTGCTTAGACCACCAGCAT 1230

C C P Q G F K C M D E G Y C Q K G D R M  
TGCTGCCCTCAGGGTTTCAAATGTATGGATGAGGGTACTGTGAGAAGGGAGACAGAATG 1290

V A G L E K M P V R Q T T L L Q H S D I  
GTGGCTGGCCTGGAGAAGATGCCTGTCCGCCAGACAACTCTGCTCCAACATGGAGATATT 1350

G C D Q H T S C P V G Q T C C P S L K G  
GGTTGTGACCAGCATACCAGCTGCCCAGTAGGGCAAACATGCTGCCCCAAGCCTGAAGGGA 1410

S W A C C Q L P H A V C C E D R Q H C C  
AGTTGGGCCTGCTGCCAGTTGCCCATGCTGTGTGCTGTGAGGACCGGCAGCACTGTTGC 1470

P A G Y T C N V K A R T C E K D A G S V  
CCGGCTGGGTACACCTGCAACGTGAAGGCGAGAACCTGTGAGAAGGATGCAGGCTCTGTC 1530

Q P S M D L T F G S K V G N V E C G A G  
CAGCCTTCCATGGACCTGACCTTTGGCTCTAAGGTTGGGAATGTGGAATGTGGTGCCGGA 1590

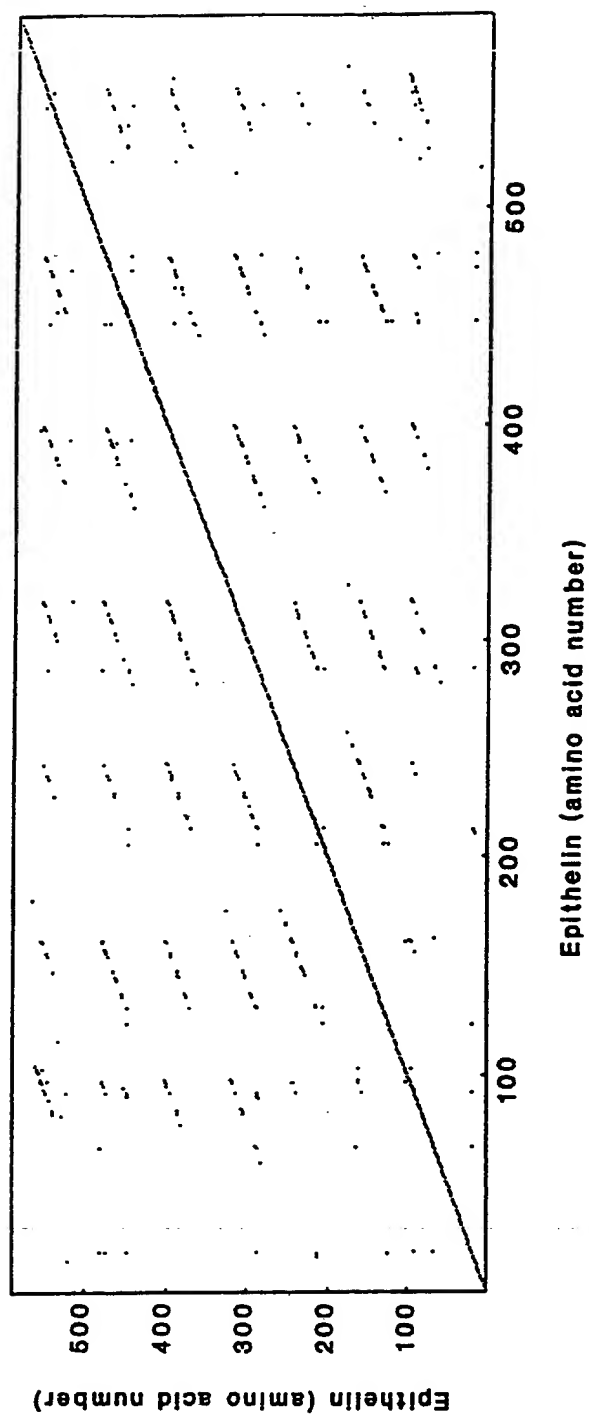
H F C H D N Q S C C K D S Q G G W A C C  
CATTTCTGCCATGATAACCAGTCCTGTTGTAAAGACAGCCAAGGAGGCTGGGCCTGCTGT 1650

P Y V K G V C C R D G R H C C P I G F H  
CCCTATGTAAAGGGTGTCTGCTGTAGAGATGGACGTCACTGTTGTCCCATTGGCTTCCAC 1710

C S A K G T K C L R K K T P R W D I L L  
TGTTCAGCCAAGGGAACCAAGTGTTTGCGGAAGAAGACCCCTCGCTGGGACATACTTTTG 1770

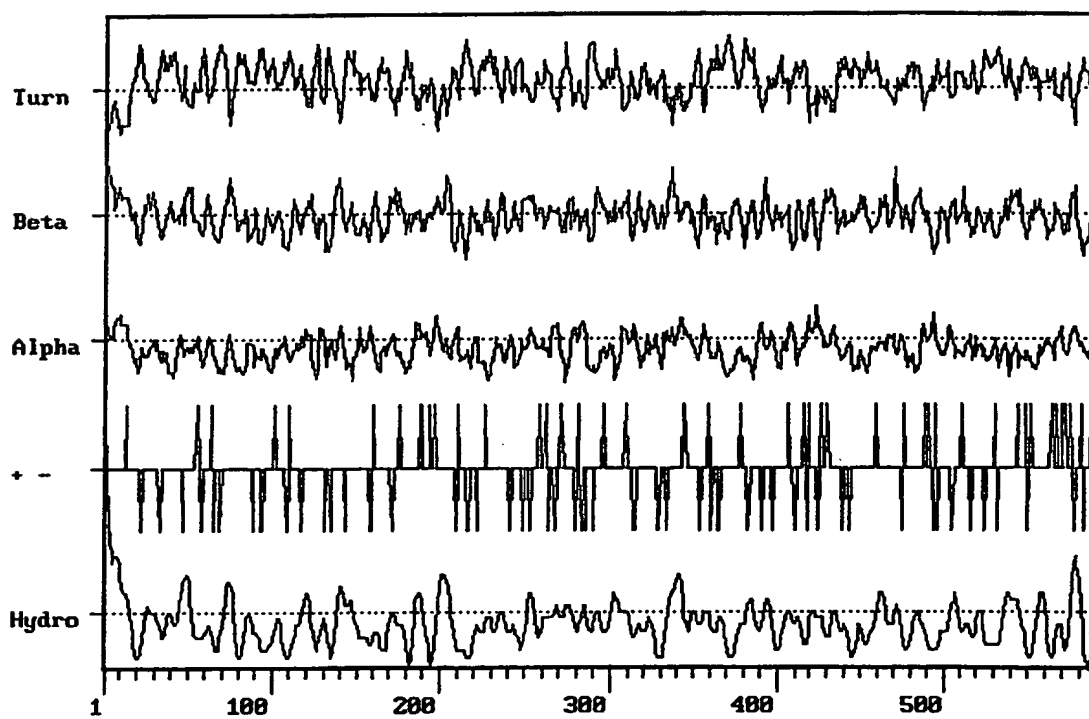
R D P A P R P L L  
AGGGATCCAGCCCCAAGACCGCTACTG 1797

Figure 19



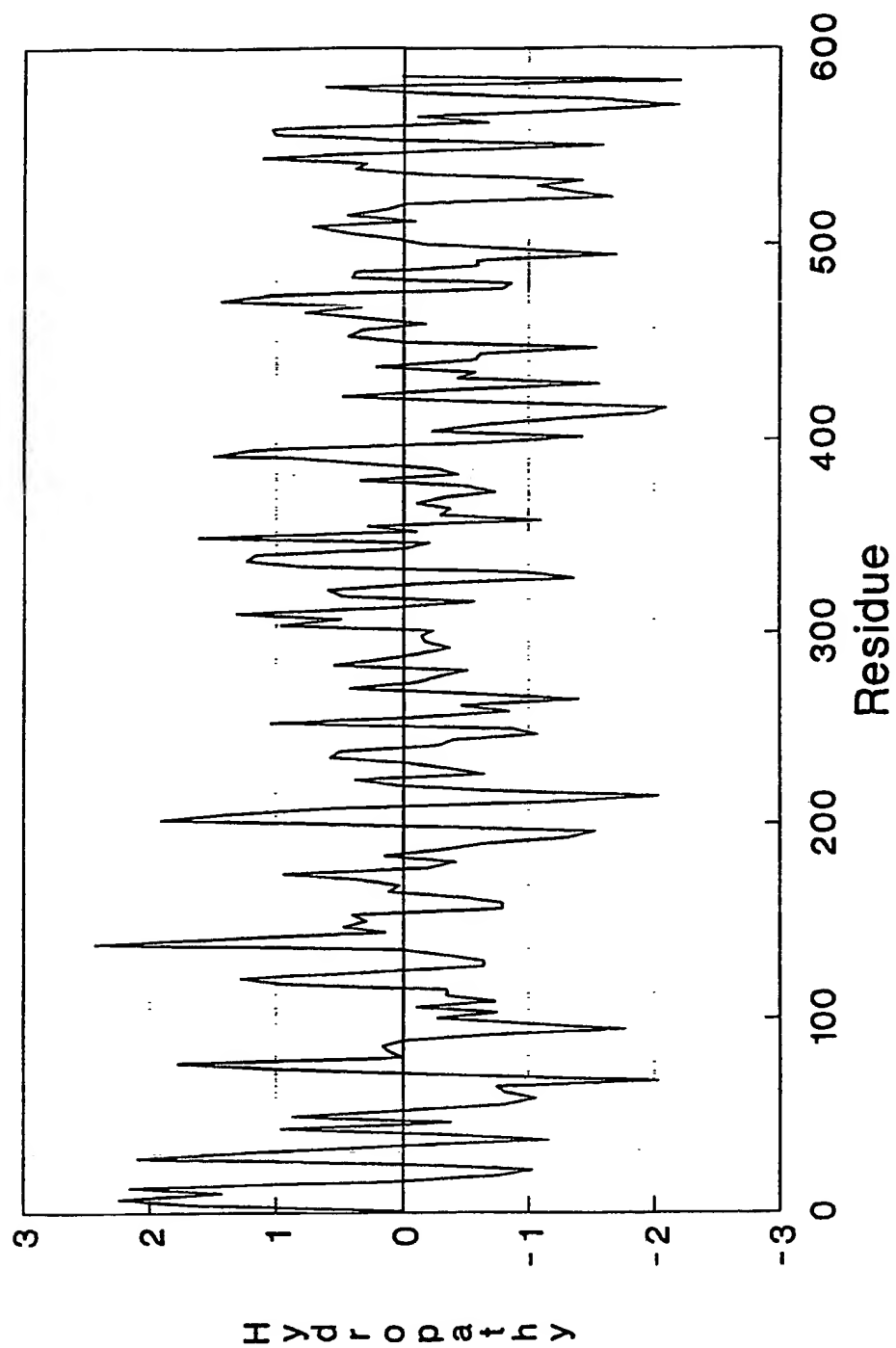
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FIGURE 20A



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FIGURE 20B



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Figure 21A

Human	..T....V..T.G.....R.....P...S....R....K..TTL..H.G	.P	60
Rat	..VDA..SA.H..IF.....P.A.A.G..H.....R..F.	GS	60
Mouse	..V.M....FA.G.....R...HH..	..	60
Human	..VDA..SA.H..IF.....P.A.A.G..H.....R..F.	RSGNNS	120
Rat	CQIRDHCPDGYSCLLTVSGTSSCCPFSEGVSCDDGQHCCPRGFHCSADGKSCSQ	-ISDSL	119
Mouse	..THG...A.....K....G..Y....Q.....F.	-M..NP	119
Human	V..I...D.....FS...V.V.....F.....T		180
Rat	LGA VQCPGSQFECPSATCCIMIDGSWGCCPMPQASCCEDRVHCCPHGASCDLVHTRCIS		179
Mouse	.....V.....V.		179
Human	.....A..L.....LSS.	.M....RSR...G.....S.....T..	240
Rat	PTGTHPLLKKFPAQRTNRAVAFPF	VVCPDAKTQCPDDSTCCELPTGKYGCCPMPNAICC	239
Mouse	.....T.....K.....SL...		239
Human	.....L.	.ENA....L....AHT.GD	300
Rat	SDHLHCCPQDTVCDLIQSKCIS	KDY-TTDLMTKLPGYPVNE	298
Mouse	.....L.	.N.-....L.....K.	298
Human	S.....Q.....T.D.QK....Q	.PH.....E.AP.H.....A.	360
Rat	TGAWGCCPFTKAVCCEDHIHCCPAGFQCHTETGTCEL	GVLPVPMKKVTASLSLPDPQIL	358
Mouse	.....A.....K....M	.I.....I.PRR.....	358
Human	.R. ....NV.....SD...Q.T.E.....S↓.....YT.VA..Q..R	.S	420
Rat	KND VPCDDFSSCPSNNTCCRLSSGDWGCCIPEAVCCLDHQHCCPQGFKCMDEGYCQK	GD	418
Mouse	.S. T.....TR..T.....K.N.....S.N.....T.LAQ.....	..	418
Human	EI.....A.RAS.SHPR.	.....G.....↓...	480
Rat	RMVAGLEKMPVRQTLLQHGD	IGCDQHTSCPVGQTCCPSLKGSWACCQLPHAVCCEDRQH	478
Mouse	T.....I.A....P..I..		478
Human	.....S...	EVV.A..ATF.ARSPH..VKD	540
Rat	CCPAGYTCNVKARTCEK	DAGSVQPSMDLTFGSKVG--N	536
Mouse	.....VDFI..PVL..L.P....--	.....E.....T....A.V	536
Human	.....RQ....A.R.....A..R.A.R.....	REA....AP.....L.Q..	593
Rat	WACCPYVKGVCRRDGRHCCPIGFHCSAKGTKCLR	KKTPRWDILLRDPAPRPLL	589
Mouse	.....L.....G.....R.....	..I....MF....V.....	589

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Figure 21B

Rat 1	GSC-QIRDHCPDGYSCLLTVSGTSSCCPFSEGVSCDDGQHCCPRGFHCSADGKSCSQ
Rat 2	VQCPGSQFECPSATCCIMIDGSWGCPMPQASCCEDRVHCCPHGASCDLVHTRCIS
II Rat 3	VVCPDAKTQCPDDSTCCELPTGKYGCCPMPNAICCS DHLHCCPQDTVCDLIQSKCIS
I Rat 4	VKC-DLEVSCP DGYTCCRLNTGAWGCCPFTKAVCCEDHIHCCPAGFQCHTETGTCEL
Rat 5	VPC-DDFSSCPSNNTCCRLSSGDWGCCPIEAVCCLDHQHCCPQGFKCMDEG-YCQK
Rat 6	IGC-DQHTSCPVGQTCCPSLKGSWACCQLPHAVCCEDRQHCCPAGYTCNVKARTCEK
Rat 7	VEC-GAGHFCHDNQSCCKDSQGGWACCPYVKGVCCRDGRHCCPIGFHCSAKGTKCLR
Mouse 1	GSC-QTHGHCPAGYSCLLTVSGTSSCCPFSGVSCGDGYHCCPQGFHCSADGKSCFQ
Mouse 2	VQCPGSQFECPSATCCIMVDGSWGCPMPQASCCEDRVHCCPHGASCDLVHTRCVS
Mouse 3	VVCPDAKTQCPDDSTCCELPTGKYGCCPMPNAICCS DHLHCCPQDTVCDLIQSKCLS
Mouse 4	VKC-DMEVSCPEGYTCCRLNTGAWGCCPFAKAVCCEDHIHCCPAGFQCHTEKGTCEM
Mouse 5	TPC-DDFTRCPTNNTCCKLNSGDWGCCPIEAVCCSDNQHCCPQGFCLAQG-YCQK
Mouse 6	IGC-DQHTSCPVGQTCCPSLKGSWACCQLPHAVCCEDRQHCCPAGYTCNVKARTCEK
Mouse 7	VEC-GEGHFCHDNQTCCCKDSAGVWACCPYLKGVCCRDGRHCCPGGFHCSARGTKCLR
Human 1	GPC-QVDAHCSAGHSCIFTVSGTSSCCPFPEAVACGDGHHCCPRGFHCSADGRSCFQ
Human 2	IQCPDSQFECPDFSTCCVMVDGSWGCPMPQASCCEDRVHCCPHGAFCDLVHTRCIT
Human 3	VMCPDARSRCPDGSTCCELPSGKYGCCPMPNATCCSDHLHCCPQDTVCDLIQSKCLS
Human 4	VKC-DMEVSCP DGYTCCRLQSGAWGCCPFTQAVCCEDHIHCCPAGFTCDTQKGTCEQ
Human 5	VPC-DNVSSCPSDTCCLTSGEWGCCPIEAVCCSDHQHCCPQGYTCVAEG-QCQR
Human 6	IGC-DQHTSCPVGQTCCPSLGGSWACCQLPHAVCCEDRQHCCPAGYTCNVKARSCEK
Human 7	VEC-GEGHFCHDNQTCCRDNRQGWACCPYRQGVCCADRRHCCPAGFRCAARGTKCLR

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Figure 21C





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Figure 21D

Tomato Protease	T	E	C	-	D	E	Y	S	Q	C	A	V	G	T	T	C	C	C	I	L	Q	F	R	R	S	C	F	S	W	G	C	C	P
Epithelin 1	V	K	C	-	D	L	E	V	S	C	P	D	G	Y	T	C	C	-	-	-	-	-	R	L	N	T	G	A	W	G	C	C	P
Epithelin 2	V	V	C	P	D	A	K	T	Q	C	P	D	D	S	T	C	C	-	-	-	-	-	E	L	P	T	G	K	Y	G	C	C	P

Tomato Protease	L	E	G	A	T	C	C	E	D	H	Y	S	C	C	P	H	D	Y	P	I	C	N	V	R	Q	G	T	C	S	M
Epithelin 1	F	T	K	A	V	C	C	E	D	H	I	H	C	C	P	A	G	F	-	Q	C	H	T	E	T	G	T	C	E	L
Epithelin 2	M	P	N	A	I	C	C	S	D	H	L	H	C	C	P	Q	D	T	-	V	C	D	L	I	Q	S	K	C	I	S

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Figure 22

M W T L V S W V A L T A G L V A G T R C  
ATGTGGACCCTGGTGAGCTGGGTGGCCTTAACAGCAGGGCTGGTGGCTGGAACGCGGTGC 100

P D G Q F C P V A C C L D P G G A S Y S  
CCAGATGGTCAGTTCTGCCCTGTGGCCTGCTGCCTGGACCCCGGAGGAGCCAGCTACAGC 160

C C R P L L D K W P T T L S R H L G 'G P  
TGCTGCCGTCCCCTTCTGGACAAATGGCCCACAACACTGAGCAGGCATCTGGGTGGCCCC 220

C Q V D A H C S A G H S C I F T V S G T  
TGCCAGGTTGATGCCCACTGCTCTGCCGGCCACTCCTGCATCTTTACCGTCTCAGGGACT 280

S S C C P F P E A V A C G D G H H C C P  
TCCAGTTGCTGCCCCCTTCCAGAGGCCGTGGCATGCGGGGATGGCCATCACTGCTGCCCA 340

R G F H C S A D G R S C F Q R S G N N S  
CGGGGCTTCCACTGCAGTGACAGACGGGCGATCCTGCTTCCAAAGATCAGGTAACAACCTCC 400

V G A I Q C P D S Q F E C P D F S T C C  
GTGGGTGCCATCCAGTGCCCTGATAGTCAGTTTGAATGCCCGGACTTCTCCACGTGCTGT 460

V M V D G S W G (C C) P M P Q A S (C C) E D  
GTTATGGTCGATGGCTCCTGGGGGTGCTGCCCCATGCCCCAGGCTTCTGCTGTGAAGAC 520

R V H C C P H G A F C D L V H T R C I T  
AGGGTGCACTGCTGTCCGCACGGTGCCCTTCTGCGACCTGGTTTACACCCGCTGCATCACA 580

P T G T H P L A K K L P A Q R T N R A V  
CCCACGGGCACCCACCCCTGGCAAAGAAGCTCCCTGCCAGAGGACTAACAGGGCAGTG 640

A L S S S V M C P D A R S R C P D G S T  
GCCTTGTCCAGCTCGGTGATGTGTCCGGACGCACGGTCCCGGTGCCCTGATGGTTCTACC 700

C C E L P S G K Y G C C P M P N A T C C  
TGCTGTGAGCTGCCCAGTGGAAGTATGGCTGCTGCCCCAATGCCCAACGCCACCTGCTGC 760

S D H L H C C P Q D T V C D L I Q S K C  
TCCGATCACCTGCACTGCTGCCCCCAAGACACTGTGTGTGACCTGATCCAGAGTAAGTGC 820

L S K E N A T T D L L T K L P A H T V G  
CTCTCCAAGGAGAACGCTACCACGGACCTCCTCACTAAGCTGCCTGCGCACACAGTGGGG 880

D V K C D M E V S C P D G Y T C C R L Q  
GATGTGAAATGTGACATGGAGGTGAGCTGCCAGATGGCTATACCTGCTGCCGTCTACAG 940

S G A W G C C P F T Q A V C C E D H I H  
TCGGGGGCTGGGGCTGCTGCCCTTTTACCCAGGCTGTGTGCTGTGAGGACCACATACAC 1000

C C P A G F T C D T Q K G T C E Q G P H  
TGCTGTCCCGCGGGGTTTACGTGTGACACGCAGAAGGGTACCTGTGAACAGGGGCCCCAC 1060

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Figure 22 (Cont.)

Q V P W M E K A P A H L S L P D P Q A L  
CAGGTGCCCTGGATGGAGAAGGCCCCAGCTCACCTCAGCCTGCCAGACCCACAAGCCTTG 1120

K R D V P C D N V S S C P S S D T C C Q  
AAGAGAGATGTCCCTGTGATAATGTCAGCAGCTGTCCCTCCTCCGATACCTGCTGCCAA 1180

L T S G E W G C C P I P E A V C C S D H  
CTCACGTCTGGGGAGTGGGGCTGCTGTCCAATCCCAGAGGCTGTCTGCTGCTCGGACCAC 1240

Q H C C P Q G Y T C V A E G Q C Q R G S  
CAGCACTGCTGCCCCAGGGCTACACGTGTGTAGCTGAGGGGCAGTGTCTCAGCGAGGAAGC 1300

E I V A G L E K M P A R R A S L S H P R  
GAGATCGTGGCTGGACTGGAGAAGATGCCTGCCCCGCCGGGCTTCCTTATCCCACCCAGA 1360

D I G C D Q H T S C P V G Q T C C P S L  
GACATCGGCTGTGACCAGCACACCAGCTGCCCCGGTGGGGCAGACCTGCTGCCCCGAGCCTG 1420

G G S W A C C Q L P H A V C C E D R Q H  
GGTGGGAGCTGGGCCTGCTGCCAGTTGCCCCATGCTGTGTGCTGCGAGGATCGCCAGCAC 1480

C C P A G Y T C N V K A R S C E K E V V  
TGCTGCCCCGGCTGGCTACACCTGCAACGTGAAGGCTCGATCCTGCGAGAAGGAAGTGGTC 1540

S A Q P A T F L A R S P H V G V K D V E  
TCTGCCCAGCCTGCCACCTTCCTGGCCCCGTAGCCCTCACGTGGGTGTGAAGGACGTGGAG 1600

C G E G H F C H D N Q T C C R D N R Q G  
TGTGGGGAAGGACACTTCTGCCATGATAACCAGACCTGCTGCCGAGACAACCGACAGGGC 1660

W A C C P Y R Q G V C C A D R R H C C P  
TGGGCCTGCTGTCCCTACCGCCAGGGCGTCTGTTGTGCTGATCGGCGCCACTGCTGTCCT 1720

A G F R C A A R G T K C L R R E A P R W  
GCTGGCTTCGCTGCGCAGCCAGGGGTACCAAGTGTGCGCAGGGAGGCCCCGCGCTGG 1780

D A P L R D P A L R Q L L  
GACGCCCCTTTGAGGGACCCAGCCTTGAGACAGCTGCTG 1819

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Figure 23

M W V L M S W L A F A A G L V A G T Q C  
ATGTGGGTCCTGATGAGCTGGCTGGCCTTCGCGGCAGGGCTGGTAGCCGGAACACAGTGT 67

P D G Q F C P V A C C L D Q G G A N Y S  
CCAGATGGGCAGTTCTGCCCTGTTGCCTGCTGCCTTGACCAGGGAGGAGCCAACACTACAGC 127

C C N P L L D T W P R I T S H H L D G S  
TGCTGTAAACCCTCTTCTGGACACATGGCCTAGAATAACGAGCCATCATCTAGATGGCTCC 187

C Q T H G H C P A G Y S C L L T V S G T  
TGCCAGACCCATGGCCACTGTCCTGCTGGCTATTCTTGTCTTCTCACTGTGTCTGGGACT 247

S S C C P F S K G V S C G D G Y H C C P  
TCCAGCTGCTGCCCGTTCTCTAAGGGTGTGTCTTGTGGTGATGGCTACCACTGCTGCCCC 307

Q G F H C S A D G K S C F Q M S D N P L  
CAGGGCTTCCACTGTAGTGCAGATGGGAAATCCTGCTTCCAGATGTCAGATAACCCCTTG 367

G A V Q C P G S Q F E C P D S A T C C I  
GGTGCTGTCCAGTGTCTCTGGGAGCCAGTTTGAATGTCCTGACTCTGCCACCTGCTGCATT 427

M V D G S W G C C P M P Q A S C C E D R  
ATGGTTGATGGTTTCGTGGGGATGTTGTCCCATGCCCCAGGCCTCTTGCTGTGAAGACAGA 487

V H C C P H G A S C D L V H T R C V S P  
GTGCATTGCTGTCCCCATGGGGCCTCCTGTGACCTGGTTACACACGATGCGTTTCACCC 547

T G T H T L L K K F P A Q K T N R A V S  
ACGGGCACCCACACCCTACTAAAGAAGTTCCCTGCACAAAGACCAACAGGGCAGTGTCT 607

L P F S V V C P D A K T Q C P D D S T C  
TTGCCCTTTTTCTGTGCTGTGCCCTGATGCTAAGACCCAGTGTCCCGATGATTCTACCTGC 667

C E L P T G K Y G C C P M P N A I C C S  
TGTGAGCTACCCACTGGGAAGTATGGCTGCTGTCCAATGCCCAATGCCATCTGCTGTTCC 727

D H L H C C P Q D T V C D L I Q S K C L  
GACCACCTGCACTGCTGCCCCCAGGACACTGTATGTGACCTGATCCAGAGTAAGTGCCTA 787

S K N Y T T D L L T K L P G Y P V K E V  
TCCAAGAACTACACCACGGATCTCCTGACCAAGCTGCCTGGATACCCAGTGAAGGAGGTG 847

K C D M E V S C P E G Y T C C R L N T G  
AAGTGCACATGGAGGTGAGCTGCCCTGAAGGATATACCTGCTGCCGCCTCAACACTGGG 907

A W G C C P F A K A V C C E D H I H C C  
GCCTGGGGCTGCTGTCCATTGCCAAGGCCGTGTGTTGTGAGGATCACATTATTGCTGC 967

P A G F Q C H T E K G T C E M G I L Q V  
CCGGCAGGGTTTCAGTGTACACAGAGAAAGGAACCTGCGAAATGGGTATCCTCCAAGTA 1027

Figure 23 (Cont.)

P W M K K V I A P R R L P D P Q I L K S  
CCCTGGATGAAGAAGGTCATAGCCCCCGCCGCTGCCAGACCCACAGATCTTGAAGAGT 1087

D T P C D D F T R C P T N N T C C K L N  
GATACACCTTGTGATGACTTCACTAGGTGTCCTACAAACAATACCTGCTGCAAACTCAAT 1147

S G D W G C C P I P E A V C C S D N Q H  
TCTGGGGACTGGGGCTGCTGTCCCATCCCAGAGGCTGTCTGCTGCTCAGACAACCAGCAT 1207

C C P Q G F T C L A Q G Y C Q K G D T M  
TGCTGCCCTCAGGGCTTCACATGTCTGGCTCAGGGGTACTGTGAGAAGGGAGACACAATG 1267

V A G L E K I P A R Q T T P L Q I G D I  
GTGGCTGGCCTGGAGAAGATACTGCCCGCCAGACAACCCGCTCCAAATTGGAGATATC 1327

G C D Q H T S C P V G Q T C C P S L K G  
GGTTGTGACCAGCATAACCAGCTGCCCAGTAGGGCAAACCTGCTGCCCAAGCCTCAAGGGA 1387

S W A C C Q L P H A V C C E D R Q H C C  
AGTTGGGCCTGCTGCCAGCTGCCCCATGCTGTGTGCTGTGAGGACCGGCAGCACTGTTGC 1447

P A G Y T C N V K A R T C E K D V D F I  
CCGGCCGGGTACACCTGCAATGTGAAGGCGAGGACCTGTGAGAAGGATGTCGATTTTATC 1507

Q P P V L L T L G P K V G N V E C G E G  
CAGCCTCCCGTGCTCCTGACCCTCGGCCCTAAGGTTGGGAATGTGGAGTGTGGAGAAGGG 1567

H F C H D N Q T C C K D S A G V W A C C  
CATTTCTGCCATGATAACCAGACCTGTTGTAAAGACAGTGCAGGAGTCTGGGCCTGCTGT 1627

P Y L K G V C C R D G R H C C P G G F H  
CCCTACCTAAAGGGTGTCTGCTGTAGAGATGGACGTCACTGTTGCCCCGGTGGCTTCCAC 1687

C S A R G T K C L R K K I P R W D M F L  
TGTTTCAGCCAGGGGAACCAAGTGTGCGAAAGAAGATTCTCGCTGGGACATGTTTTTG 1747

R D P V P R P L L  
AGGGATCCGGTCCCAAGACCGCTACTG 1774

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Figure 24

K W G C C P M P N A I C C S D H L H C C AAGTGGGGGTGTGCCCCGATGCCCAATGCCATTTGCTGCTCCGACCACCTGCACTGCTGC	60
P Q N T V C D L T Q S K C L S K E N A T CCCCAGAACACTGTGTGTGACCTGACCCAGAGTAAGTGCCCTCTCCAAGGAGAACGCTACG	120
D L L T K L P A H T V Q D V K C D M E V GACCTCCTCACCAAGCTGCCCCGACACACAGTGCAAGTGCAGGATGTCAAGTGCACATGGAGGTG	180
S C P D D Y T C C R L Q S G A W G C C P AGCTGCCCAGACGACTACACCTGCTGCCGCTACAGTCCGGGGCTGGGGCTGCTGCCCT	240
F V Q A V C C E D H V H C C P S G F R C TTTGTGCAGGCCGTGTGCTGTGAGGACCATGTGCACTGCTGCCCGTCCGGGTTTAGGTGT	300
D T E K G V C E Q G T R Q V P W M K K A GACACAGAGAAGGGTGTGTGTGAGCAGGGGACCCGCCAGGTGCCGTGGATGAAGAAAGCC	360
P A H L S L L D L G A V E G D V P C D N CCAGCCCACCTCAGCCTGCTGGACCTCGGAGCAGTGGAGGGGACGTCCCTGTGATAAC	420
V T S C P S S T T C C R L K S G E W A C GTCACCAGCTGTCTTCTTCCACTACCTGCTGTGCACTCAAGTCTGGGGAGTGGGCCTGC	480
C P A P E A V C C S D H Q H C C P Q D TGCTCTGCTCCAGAGGTGTCTGCTGCTCGGACCACCAGCACTGCTGTCCCCAAGATAC	539

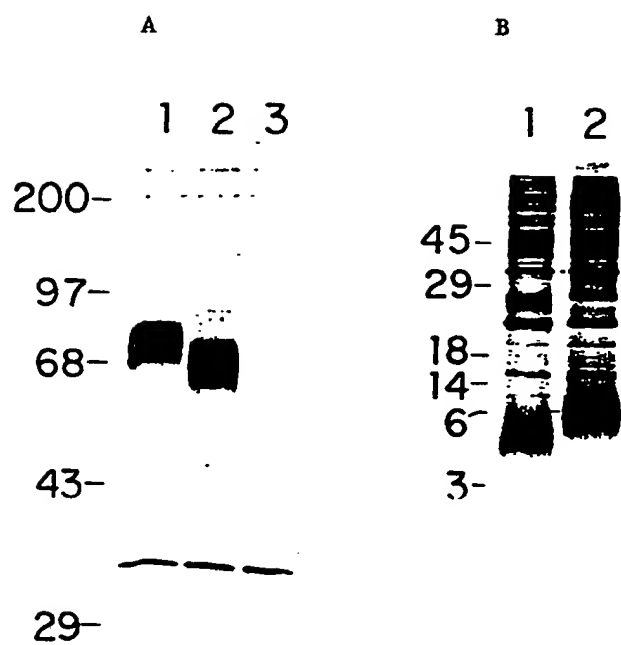
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Figure 25

K W G C C P M P X G V C C R D E E H C C	
AAGTGGGGTTGTTGCCCCATGCCGXGAGGCGTGTGCTGCCGGGATGAGGAGCACTGCTGT	60
P H S T S C D L E R G R C V S P T G D V	
CCCCACTCCACCAGCTGTGATTTGGAGCGCGGGCGCTGTGTGTCCCTACGGGGGACGTC	120
P M A T K F P A W K R P R G A A A Q P R	
CCCATGGCCACCAAATTCCCGGCCTGGAAGAGACCGCGGGTGCTGCGGCACAGCCCCGG	180
L R V P A V V G D V K C D D E H S C P D	
CTCCGCGTCCCAGCAGTGGTTGGTGACGTGAAGTGTGACGATGAGATGAGCTGTCCCGAC	240
G N T C C R L S S G Q W G C C P L E Q A	
GGGAACACGTGCTGCAGGCTGAGCTCCGGGCAGTGGGGGTGCTGCCCGCTGGAGCAGGCC	300
V C C P D H I H C C P Q D	
GTGTGCTGCCCCGACCACATCCACTGCTGCCCCCAAGATAC	341

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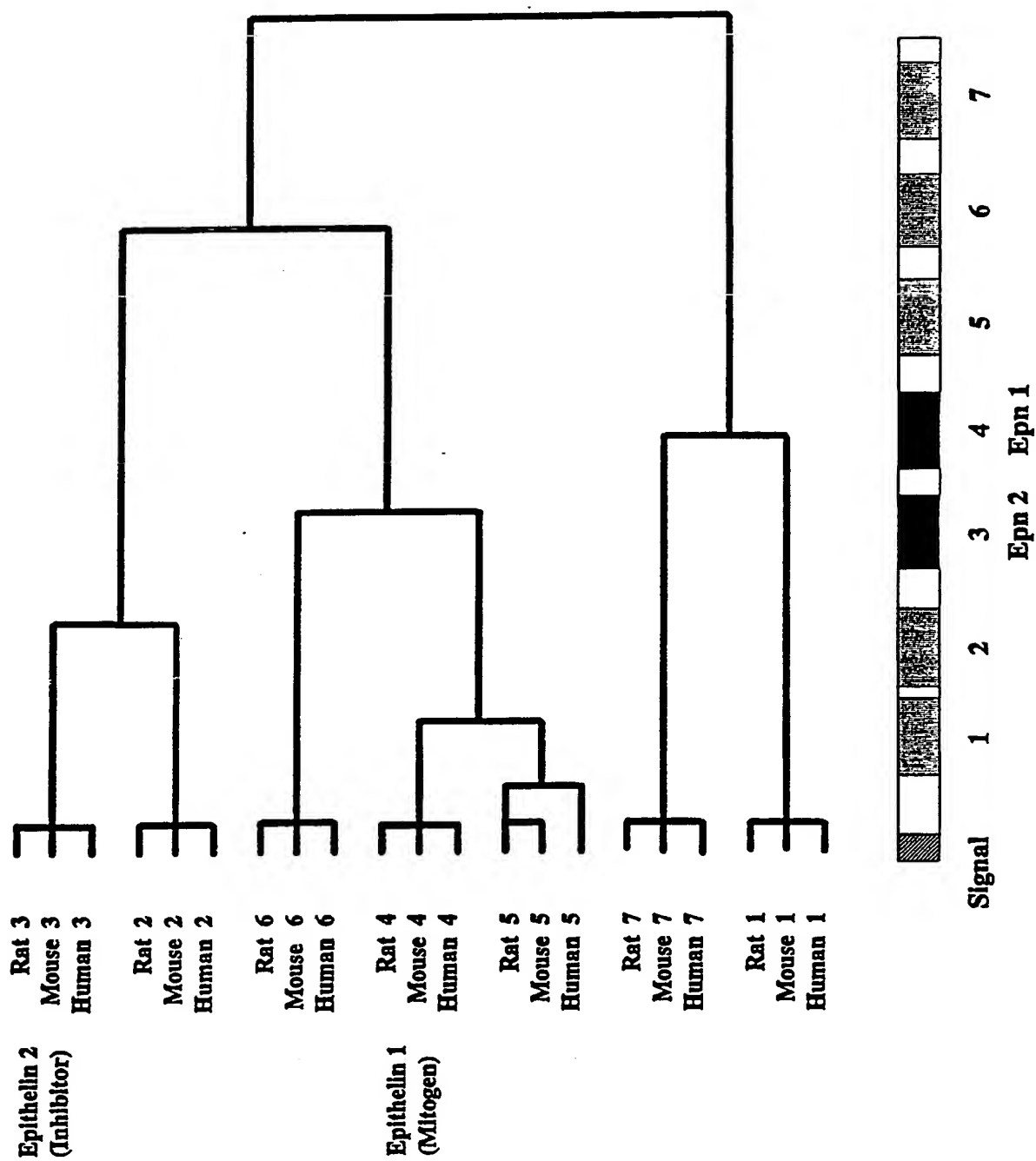
Figure 26





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Figure 27



# INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US91/02321**

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC <b>IPC(5):</b> C07K 7/10, 13/00; C12N 15/18 <b>U.S.C1.:</b> 530/324, 299; 536/27; 514/12														
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched <sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="padding: 5px; vertical-align: top;">US:</td> <td style="padding: 5px; vertical-align: top;">530/324, 399; 536/27; 514/12;</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup></div> <p>Automated Patent Search, Sequence Data banks, Chemical Abstracts Service</p>			Classification System	Classification Symbols	US:	530/324, 399; 536/27; 514/12;								
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US:	530/324, 399; 536/27; 514/12;													
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category <sup>10</sup></th> <th style="border-bottom: 1px solid black;">Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 15%; border-bottom: 1px solid black;">Relevant to Claim No. <sup>13</sup></th> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">SCIENCE, Volume 243, issued 24 February 1989, Shoyab et al., "Structure and Function of Human Amphiregulin: A Member of the Epidermal Growth Factor Family", pages 1074-1076.</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-51</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">SCIENCE, Volume 234, issued 10 October 1986, Rozengurt, "Early Signals in the Mitogenic Response", pages 161-166. See Table 1, page 162.</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-51</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">US,A, 4,590,003 (Twardzik et al.) 20 May 1986, see entire document.</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-51</td> </tr> </table>			Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	A	SCIENCE, Volume 243, issued 24 February 1989, Shoyab et al., "Structure and Function of Human Amphiregulin: A Member of the Epidermal Growth Factor Family", pages 1074-1076.	1-51	A	SCIENCE, Volume 234, issued 10 October 1986, Rozengurt, "Early Signals in the Mitogenic Response", pages 161-166. See Table 1, page 162.	1-51	A	US,A, 4,590,003 (Twardzik et al.) 20 May 1986, see entire document.	1-51
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"d" document member of the same patent family</p> </div> </div>														
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; vertical-align: top; padding: 5px;">           Date of the Actual Completion of the International Search  <b>27 June 1991</b> </td> <td style="width: 50%; vertical-align: top; padding: 5px;">           Date of Mailing of this International Search Report  <div style="text-align: center; font-weight: bold; font-size: 1.2em;">13 AUG 1991</div> </td> </tr> <tr> <td style="vertical-align: top; padding: 5px;">           International Searching Authority  <b>ISA/US</b> </td> <td style="vertical-align: top; padding: 5px;">           Signature of Authorized Officer  <b>Nina Ossanna Ph.D.</b> </td> </tr> </table>			Date of the Actual Completion of the International Search <b>27 June 1991</b>	Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold; font-size: 1.2em;">13 AUG 1991</div>	International Searching Authority <b>ISA/US</b>	Signature of Authorized Officer <b>Nina Ossanna Ph.D.</b>								
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